A Murine Model for Haemangioblast Transplantation

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If

If you can keep your head when all about you
Are losing theirs and blaming it on you,
If you can trust yourself when all men doubt you,
But make allowance for their doubting too;
If you can wait and not be tired by waiting,
Or being lied about, don't deal in lies,
Or being hated, don't give way to hating,
And yet don't look too good, nor talk too wise:

If you can dream - and not make dreams your master,
If you can think - and not make thoughts your aim;
If you can meet with Triumph and Disaster
And treat those two impostors just the same;
If you can bear to hear the truth you've spoken
Twisted by knaves to make a trap for fools,
Or watch the things you gave your life to, broken,
And stoop and build 'em up with worn-out tools:

If you can make one heap of all your winnings
And risk it all on one turn of pitch-and-toss,
And lose, and start again at your beginnings
And never breath a word about your loss;
If you can force your heart and nerve and sinew
To serve your turn long after they are gone,
And so hold on when there is nothing in you
Except the Will which says to them: "Hold on!"

If you can talk with crowds and keep your virtue,
Or walk with kings - nor lose the common touch,
If neither foes nor loving friends can hurt you,
If all men count with you, but none too much;
If you can fill the unforgiving minute
With sixty seconds' worth of distance run,
Yours is the Earth and everything that's in it,
And - which is more - you'll be a Man, my son!

Rudyard Kipling (1865-1936)

Declaration

I declare that I have written this thesis based on my own work and that any contributions made by others have been clearly indicated. The work contained in this thesis has not been submitted for any other degree, diploma or professional qualification.

Yasmin Babaie, 2004

Abstract

The haemangioblast is believed to be the common precursor of haematopoietic cells, the endothelial cells that form the vessels as well as the mural cells associated with the vessel endothelium. Despite its suggested existence, such a cell has eluded discovery and isolation *in vivo*. Its existence has been inferred however, by *in vitro* differentiation and analysis of embryonic stem (ES) cells. The possibility of generating haemangioblastic progenitors from ES cells may have therapeutic applications.

The *flk-1* gene is believed to be a key marker of the haemangioblast. *Flk-1* is a receptor tyrosine kinase known to be expressed on a subset of mesodermal cells designated to form the vasculature. Its role is believed to be to ensure the migration of haemangioblastic precursors to the appropriate sites for further proliferation and differentiation to the various vascular lineages.

A promoter trap strategy was used to target a single allele of the *flk-1* gene with one of two reporter constructs. One placed the EGFP reporter under *flk-1* transcriptional control, while the other transcribed the selectable HPRT enzyme, which could be used to isolate *flk-1* expressing cells in the HM1 *hprt* deficient ES cell line.

Homologous recombination was successfully achieved with both targeting vectors. After initially promising results indicating that all targeted lines were appropriately expressing the reporter, silencing of the GFP reporter was observed in both of the successfully isolated targeted lines. The cause of silencing could not be deduced although as both targeted lines were silenced, the design of the targeting vector may have been responsible. Four HPRT targeted selectable lines were successfully isolated. All lines showed appropriate transgene expression at high enough levels for effective HAT selection to isolate pure populations of *flk-1* expressing cells.

Two methods of deriving haemangioblastic progenitors from ES cells were tested for their efficacy in the isolation of a pure haemangioblast population based on the

expression of *flk-1*. Methylcellulose cultures were found to be highly variable and problematic to carry out successfully whereas monolayer differentiation on collagen IV was more consistent. This system was then used to optimise the differentiation and selection regime applied to the *flk-1*/HPRT targeted cells in an attempt to isolate a pure haemangioblast progenitor population.

Flk-1/HPRT targeted cells were marked with a constitutive GFP transgene, differentiated and selected for *flk-1*/HPRT expression on collagen IV. The selected cells were injected into the blastocyst stage embryo to see whether a developmentally more advanced cell population would survive and contribute to the expected cell lineages.

Contribution of the GFP marked cells was observed in mesodermal and highly vascularised organs such as the heart, liver and kidney but was absent from the ectodermally derived brain. Expression was strongly localised around regions of vascularisation but further transplantation experiments are required to assess whether the injected cells were directly contributing to the vasculature, or whether they had a broader potentiality as a result of dedifferentiation, reprogramming or contamination by more primitive cells.

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To my parents Pari and Behrooz.

Thank you for the sacrifices you have made so that I could have a better life.

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List of Abbreviations

>	greater than
acLDL	acetylated low density lipoprotein
AGM	aorta gonad mesonephros
ASC	adult stem cell
bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix
BL-CFC	blast colony forming cell
bp	base pairs
BSA	bovine serum albumen
$CaCl_2$	calcium chloride
cDNA	complementary deoxyribonucleic acid
CIP	calf intestinal alkaline phosphatase
CMV	cytomegalovirus
DAPI	2-(4-amidinophenyl)-6-indolecarbamidinedihydrochloride
dCTP	2'-deoxycytidine-5'triphosphate
ddH_2O	distilled and deionised water
DEPC	diethyl pyrocarbonate
dH_2O	deionised water
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
dpc	days *post coitum*
DPX	distyrene, tricresyl phosphate, xylene
DTT	dithiothreitol
EB	embryoid body
EC	embryonal carcinoma
ECGS	endothelial cell growth supplement
EDTA	ethylenediaminetetraacetic acid, disodium
EG	embryonic germ
EGFP	enhanced green fluorescent protein
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
EJC	exon junction complexes
EMT	epithelium to mesenchyme transition
ES	embryonic stem
FACS	fluorescence activated cell sorting
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FLK-1	foetal liver kinase-1
FLT-1	*fms*-like kinase-1
G-CSF	granulocyte-colony stimulating factor
GFP	green fluorescent protein
GMEM	Glasgow's modified Eagle's medium
gp	glycoprotein

HAT	hypoxanthine, aminopterin, thymine selection
HBS	HEPES buffered saline
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hES	human embryonic stem
HIF-1α	hypoxia-inducible transcription factor 1α
HPRT	hypoxanthine-guanine phosphoribosyl transferase
HRP	horseradish peroxidase
HSC	haematopoietic stem cell
HSV	herpes simplex virus
HT	hypoxanthine and thymine selection
ICM	inner cell mass
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	intracytoplasmic immunoreceptor tyrosine inhibitory motif
kb	kilobase pairs
KCl	potassium chloride
KDR	kinase insert domain region (human *flk-1* gene)
KH_2PO_4	potassium dihydrogen orthophosphate
KO-DMEM	Knockout Dulbecco's modified Eagle's medium
LB	Luria Bertani
LIF	leukaemia inhibitory factor
LTR	long-term repopulating/repopulation
MAPC	multipotent adult progenitor cell
MAPK	mitogen-activated protein kinase
mES	murine embryonic stem
mg	milligram
$MgCl_2$	magnesium chloride
MIAMI	marrow-isolated adult multilineage inducible
ml	millilitre
MOPS	3-(N-morpholino)-propanesulfonic acid
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cell
MTG	monothioglycerol
Na_2HPO_4	disodium hydrogen orthophosphate
NaCl	sodium chloride
$NaH_2PO_4.H_2O$	sodium dihydrogen orthophosphate-1-hydrate
NaOH	sodium hydroxide
NBCS	newborn calf serum
neo	neomycin phosphotransferase gene
ng	nanograms
NMD	nonsense-mediated mRNA decay
NSC	neural stem cell
°C	degrees Celsius
oct-4	octamer binding protein 4
OD	optical density
PAS	para-aortic splanchnopleura

PBS	phosphate buffered saline
PCA	paraffin clearing agent
PCLP-1	podocalyxin-like protein 1
PCR	polymerase chain reaction
PDGF-BB	platelet-derived growth factor BB
PE	r-phycoerythrin
PECAM-1	platelet-endothelial cell adhesion molecule 1
PEF	primary embryonic fibroblast
PFGE	pulsed field gel electrophoresis
PGC	primordial germ cell
PGK	phosphoglycerate kinase
pH	potential of hydrogen
PlGF	placental growth factor
PTP	protein-tyrosine phosphatase
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcriptase
RT	room temperature
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase PCR
SCF	stem cell factor
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulphate
SSEA	stage-specific embryonic antigen
STAT	signal transducer and activator of transcription
TAE	tris-acetate-EDTA
TBE	tris-borate-EDTA
TE	tris-EDTA
TEG	trypsin EGTA
T_m	melting temperature
Tris	tris(hydroxymethyl)aminomethane
Tween20	polyoxyethylenesorbitan monolaurate
U	unit
UV	ultraviolet
v/v	volume per volume
VEGF	vascular endothelial growth factor
w/v	weight per volume
α-MEM	alpha modified minimum essential Eagle's medium
µF	microfarad
µg	micrograms
µl	microlitres
ρM	picomoles

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CHAPTER 1

Introduction and Background

Introduction and Background

1.1. General Introduction

Cell-based therapies are beginning to be considered as real possibilities in the treatment of a variety of disorders resulting from irreversible cell loss or degeneration, and where cell function has been lost or abrogated. Their success and range of applications has been limited by an inability to source the appropriate cell types, which will graft and proliferate, in a large enough quantity to be functionally active, whilst maintaining cell identity and cell cycle integrity. The discovery of embryonic stem (ES) cells and their pluripotency was a key step towards addressing the limitations of somatic cell therapies. Although much remains to be done to address problems of functionality, tumorigenicity and proliferative control in differentiated ES cells, prior to transplantation or *in situ* transplanted ES cells post-transplantation, some indirect studies already hint at the powerful impact of ES cell technology on disease and cellular degeneration (Kim *et al.*, 2002; Nishimura *et al.*, 2003). With the isolation (Thomson *et al.*, 1998) and successful differentiation of human ES cells to a variety of cell lineages (reviewed by Pera, 2001), ES cell transplantation as a therapeutic option has made a crucial move towards becoming a reality and research is increasingly being directed to overcome the obstacles to its success.

A model scenario for the therapeutic use of ES cells is to derive a lineage-specific progenitor population *in vitro*, which is then transplanted to the required site to graft and proliferate. The population should be free of undifferentiated ES cells that could be tumorigenic in an inappropriately controlled environment. It should also be able to respond to environmental cues to control its activity with respect to proliferation and direction of differentiation.

This project has been designed to investigate the possibility of using an *in vitro*-derived progenitor population therapeutically. The progenitor population chosen for investigation is the haemangioblast: the hypothesised precursor to all cells that comprise the vertebrate vasculature. By deriving a pure haemangioblastic population

from ES cells *in vitro*, and transplanting the cells back into embryos and tracking the progress of the cells, several questions will be addressed. It has yet to be tested whether *in vitro* differentiation protocols adequately mimic *in vivo* events. The stringent controls and complex signalling molecules' interactions, present in the developing embryo as well as the adult organism, may be beyond our scope for understanding and almost certainly beyond our abilities to replicate in culture. However it may not be necessary to replicate these exact scenarios to obtain a population of cells recognisable by the intrinsic signalling machinery of the organism in which they are transplanted. The only possible requirement is that once engrafted, a functioning cell population carrying enough of the differentiated characteristics, such as cell surface receptors and activated transcription factors, is maintained long enough to be captured by the appropriate pathways and directed to carry out its designated function.

A haemangioblastic or endothelial progenitor population could have therapeutic potential in several disorders. Endothelial dysfunction is commonly associated with atherosclerosis and the onset of coronary syndromes. Loss of endothelial function results in a loss of vasomotor control, reduction in nitric oxide production and elevated levels of inflammation and the development of a procoagulant surface (Kathir and Adams, 2003). Type 2 diabetes can also lead to endothelial dysfunction as can hypertension, both of which ultimately result in organ damage. Alzheimer's disease has also been postulated to be a microvascular disorder with neurodegenerative consequences instead of the reverse, which was previously thought to be the case (de la Torre and Stefano, 2000). Endothelial progenitors could aid the repair of the endothelial layer gaps that appear in advancing stages of Alzheimer's disease.

1.2. Aims of this Project

To utilise transgenic strategies to enable the selection of a defined haemangioblastic or endothelial progenitor population.

To test the *in vivo* viability and functionality of a purified haemangioblastic or endothelial progenitor population derived from the differentiation of ES cells.

1.2.1. Objectives

- Genetic modification of a murine ES (mES) cell line at the *flk-1* locus using gene targeting, to enable the identification and isolation of a pure population of haemangioblastic progenitors.
- Optimisation of *in vitro* differentiation protocols for the derivation of haemangioblastic precursors from mES cells.
- Isolation of a pure population of haemangioblastic precursors *in vitro.*
- Gauging the repopulation capacity of the isolated haemangioblastic progenitors and thus the ability to isolate a transplantable progenitor population *in vitro* by transplantation into murine embryos.
- Blastocyst injection of *flk-1* heterozygous knockout ES cell lines to generate chimaeric mice, and following germline transmission, the generation of heterozygous knockout mice to track haemangioblast expression. (The ultimate intention was to discover whether an ES cell-derived haemangioblastic population is sufficient to rescue a lethal gene knockout that prevents the development of intrinsic haemangioblasts).

1.3. Background

1.3.1. Embryonic Carcinoma Cells

Teratomas are tumours that consist of a disorganised mass of differentiated cells and tissues derived from any or all of the three germ layers (Solter and Damjanov, 1979; Martin, 1980; Mintz and Fleischman, 1981). They are referred to as germ cell tumours due to their being commonly found in the gonads (Hooper, 1991).

The differentiated cells comprising the tumour arise from a pluripotent and malignant cell population of embryonic carcinoma (EC) cells. The differentiated cells are benign and it is the activity of residual EC cells that are the cause of malignant teratomas, also known as teratocarcinomas. Teratocarcinomas can be generated experimentally by the transplantation of genital ridges consisting of primordial germ cells (PGCs) of male embryos from certain mouse strains or the grafting of early embryos from most strains (Hooper, 1991). The strain specificity of teratogenesis in PGCs can be attributed to genetic variation between strains. However, the ability of teratomas to form from epiblast cells from most mouse strains can only be attributed to the inherent properties of the epiblast that liken them to EC cells and not to oncogenic mutations. This hinted at a close functional relationship between EC cells and a population of cells that had a similar potentiality and parallel role within a developing embryo (Stevens, 1958; Pierce and Beal, 1964). This implied that EC cells were essentially the same as the cells within the epiblast, only differing due to extrinsic influences and stimuli they received from their microenvironment. This was supported by observations that EC cells contributed to many tissues in the development of embryos without disrupting the normal course of differentiation and tissue formation when transplanted into an embryo.

The growing understanding of the properties of EC cells and especially the fact that tumorigenicity was not a property of all EC cell lines emphasised the importance of the microenvironment in their isolation and maintenance. This provided the stimulus to isolate equivalent cells from the early embryo, which, when cultured *in vitro*, form ES cells.

1.3.2. Embryonic Stem Cells

1.3.2.1. Isolation of ES Cells

ES cells were first derived in 1981 using two different isolation methods (Evans and Kaufman, 1981; Martin, 1981). Evans and Kaufman cultured blastocysts after implantational delay by ovarioectomy, selecting and subculturing the inner cell mass (ICM) cells on feeder cells until an ES cell line was established. Martin isolated

ICM cells using immunosurgery which involved the lysis of the exposed trophectoderm with a readily binding antibody that could trigger the complement cascade when its constituents were added to the cells (Solter and Knowles, 1975; Martin, 1981), leaving only the ICM remaining. These ICM cells were used to establish ES lines after culture on feeder layers but also included the addition of conditioned medium from the PSA-1 EC cell line (Martin, 1981).

These two strategies have since been modified and perfected. It is now known that neither implantation delay nor the addition of conditioned medium is crucial for the success of ES cell isolation methods (as long as leukaemia inhibitory factor (LIF) is included in the isolation medium). Genetic background appears to be a key issue in the successful isolation of ES cells from murine embryos, with success being limited mostly to 129 and C57BL/6 strains. Embryonic stage seems less of an important factor with ES cells being isolated from morulae (Eistetter, 1989) as well as from early postimplantation embryos (Wells *et al.*, 1991). More recently, ES cell lines from CBA strain mice – a strain known to be refractory to ES cell isolation, have been isolated using transgenic strategies (McWhir *et al.*, 1996; Gallagher *et al.*, 2003). As well as strain specificity, there also appears to be a selective pressure in favour of male lines. This may be due to an absence of X-chromosome inactivation (Robertson *et al.*, 1983) or lack of imprinting of the X-chromosome in female ES cell lines.

ES cells have not been subjected to as abnormal a microenvironment as EC cells are subject to in tumorigenesis and they are therefore believed to mimic the natural course of embryonic development more accurately than EC cells (Beddington and Robertson, 1989). However, the exact developmental stage that corresponds to ES and EC cells is more ambiguous. The expression profile of some lines of EC cells show greater similarities to the epiblast than other cell types in the embryo (Dewey *et al.*, 1978; Martin *et al.*, 1978b; Lovell-Badge and Evans, 1980).

1.3.2.2. Obstacles to ES Cell Isolation in Other Species

Until the recent isolation of human ES (hES) cells, ES cell isolation has been limited to certain inbred strains of mice and several F1 strains. Although ES cells has been reported from a range of species such as rat (Iannaccone *et al.*, 1994), chick (Pain *et al.*, 1996), pig (Shim *et al.*, 1997), horse (Saito *et al.*, 2002), rabbit (Graves and Moreadith, 1993) and fish (Sun *et al.*, 1995), none have been proved in the generation of germline chimaeras, germline transmission being the widely accepted test of true pluripotency. HES cells cannot be tested in such a way for obvious ethical objections.

1.3.3. Properties of mES Cells

1.3.3.1. Maintenance of ES cell pluripotency

1.3.3.1.1. Leukaemia Inhibitory Factor

ES cell isolation was carried out by culturing the ICM and the resulting ES cell lines on feeder cells – typically murine foetal fibroblasts. It was discovered that the soluble factors secreted by the feeder layer were enough to maintain ES cell pluripotency and conditioned medium from the buffalo rat liver (BRL) cell line was sufficient for ES cell survival and self-renewal (Smith and Hooper, 1987). The cytokine responsible for this activity in the conditioned medium was identified as LIF (Smith *et al.*, 1988).

The LIF knockout mouse is viable and lives to adulthood although females are sterile (Stewart *et al.*, 1992). This indicates that survival of the epiblast cells is not solely dependent on the activity of LIF and that there must be pleiotropy between LIF and other members of the cytokine family to which it belongs. LIF binds to a heterodimer between the LIF receptor and the glycoprotein 130 (gp130) transmembrane receptor to maintain ES cells in the pluripotent state, reminiscent of the cells of the epiblast. Stimulation of gp130 results in the activation of signal transducer and activator of transcription 3 (Niwa *et al.*, 1998) and the MEK/ERK signalling pathway (Burdon *et al.*, 1999). In the absence of LIF, gp130 homodimers can be activated by binding interleukin 6 (IL6). *In vitro*, IL6 cannot be used in place of LIF for the maintenance of ES cells unless the IL6 receptor (which ES cells do not

express) is provided in its soluble form (Nichols *et al.*, 1994; Yoshida *et al.*, 1994). This functional redundancy may explain the non-lethal phenotype of the LIF knockout. The knockout of the LIF receptor is lethal, resulting in severe developmental abnormalities. These defects do not emerge until after implantation (Ware *et al.*, 1995). The gp130 knockout mouse also develops normally beyond the epiblast stage of development until midgestation (Yoshida *et al.*, 1996), again signifying the non-essential role of gp130-mediated signalling for the survival of the pluripotent cells in the epiblast. However murine embryos lacking gp130 do not survive past the epiblast stage if they go through diapause (a process of implantation delay seen in mice that become pregnant when they are lactating, or after severe environmental stresses) suggesting a role for gp130-mediated signalling in the maintenance of the epiblast through diapause (Nichols *et al.*, 2001).

1.3.3.1.2. Oct4

The octamer binding protein 4 (*oct4*) is a member of the POU (Pit, Oct, Unc) family of transcription factors, which have various roles in development. *Oct4* is ubiquitously expressed in the blastomeres of the early embryo before becoming restricted to the ICM and the epiblast (Pesce *et al.*, 1998). It is a key transcription factor in the maintenance of ES cell pluripotency and is essential for the formation of the ICM. Similar to LIF, *oct4* has a role in the activation of STAT3 and the maintenance of pluripotency. Unlike LIF however, the knockout is lethal and results in the failed development of pluripotential ICM (Nichols *et al.*, 1998).

1.3.3.1.3. Nanog

Human ES cells fail to respond to gp130 stimulation as is seen in mES cells. This hints at the possibility of there being alternative mechanisms that could maintain pluripotency. Recently a transcription factor called *nanog* has been identified in the ICM and in ES cells that has a role in maintaining pluripotency but which acts independently of gp130 and STAT3 signalling (Chambers *et al.*, 2003; Mitsui *et al.*, 2003). Interestingly, the *nanog* knockout phenotype was comparable with the phenotype observed when *oct4* was overexpressed: embryonic cells and ES cells differentiated to parietal endoderm; it may therefore act to suppress differentiation to

the endodermal lineage. *Nanog* was also found not to function properly in the absence of *oct4* hinting at a cooperative balance of the two transcription factors in the maintenance of pluripotency. **Figure 1.1** shows a schematic of the key genes involved in the regulation of ES cell self-renewal and the decision to differentiate.

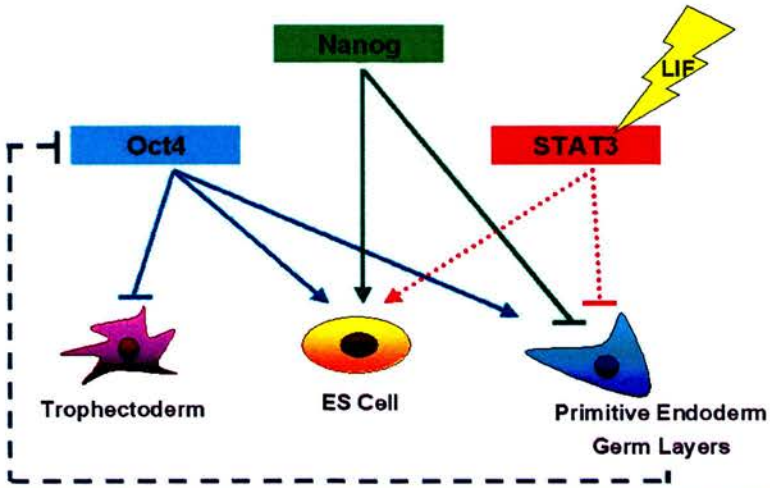

Figure 1.1 *Interactions and influences of transcription factors in the maintenance and loss of pluripotency. Oct4 and nanog are both essential for the maintenance of ES cell self-renewal. Oct4 blocks trophoblast differentiation and promotes differentiation to the endodermal lineage and the germ layers. Nanog blocks the differentiating influence of Oct4 and in so doing, also positively regulates Oct4, which is downregulated by the process of differentiation. STAT3, activated by the influence of LIF, carries out a similar role to Nanog. Figure adapted from Chambers et al. (2003).*

1.3.4. Embryonic Germ Cells

A third source of pluripotent cells can be isolated from the primordial germ cells of the genital ridge. When cultured in the presence of LIF, stem cell factor (SCF) and basic fibroblast factor (Kazemi *et al.*, 2002), they proliferate and behave as ES cells do and are known as embryonic germ (EG) cells (Matsui *et al.*, 1992; Resnick *et al.*, 1992). They have similar properties and expression patterns to ES cells and the only key difference between the two types of cells is the imprinting status of EG cells derived from latter stage PGCs (after approximately 10 days *post coitum* (dpc)) when their genomic imprinting status has been lost. As a consequence, EG cells from older embryos are unable to contribute to the germline of chimaeras (Labosky *et al.*, 1994).

1.4. Murine ES Cell Differentiation

The pluripotency of ES cells has been greatly exploited for germline modification and the generation of transgenic mice. *In vitro* differentiation has enabled the modelling of *in vivo* events during development that would otherwise be inaccessible, thus allowing complex pathways and associated signalling mechanisms to be dissected. Despite the removal of the microenvironment that would provide much of the direction for a differentiating cell, some headway has been made in the mimicry of various factors that may constitute what a differentiating cell would encounter in its *in vivo* microenvironment.

Beyond the possible oversimplification of the microenvironment, and despite their ability to contribute to germline chimaeras, it has been observed in several studies that the imprinting status of ES cells can be relaxed (Poirier *et al.*, 1991; Allen *et al.*, 1994; Szabo and Mann, 1994). The targeting of several key proto-oncogenes highlighted disparities between *in vivo* and *in vitro* phenotypes as a consequence of gene loss. *C-fos* (Field *et al.*, 1992; Hilberg and Wagner, 1992) and *c-myc* (Davis *et al.*, 1993) showed no apparent alteration of ES cell behaviour or differentiation yet the *in vivo* implications of their loss was severe and led to noticeable abnormalities. Such possibilities must be considered in the *in vitro* modelling of developmental events. ES cells may not manifest the complete range of developmental functions and requirements because of the absence of the same physical delineation of differentiated cell populations or barriers to cell signalling and differentiation. This could lead to redundancy in several signalling mechanisms allowing for the compensation of function that could not occur *in vivo*.

When cultured in suspension, in the absence of LIF, both EC and ES cells generate embryoid bodies (EBs), as well as differentiated cells from the three germ layers (Martin *et al.*, 1977; Doetschman *et al.*, 1985). In EBs (called this because of a phenotypic resemblance to the egg cylinder stage embryo (Hooper, 1991)), cells were seen to undergo compaction, similar to the morula stage of the embryo. The surface develops to comprise endodermal cells and the ball of cells eventually develops a cavity. The internal cells were seen to form a variety of differentiated

tissues. (Martin *et al.*, 1977; Doetschman *et al.*, 1985; Karbanova and Mokry, 2002). Cavitation of the EBs has been found to be a crucial step in their further differentiation. Cystic EBs form spontaneously (Evans and Kaufman, 1981; Martin, 1981). Similar cavitation is seen in the embryonic YS and is a result of apoptotic mechanisms (Doetschman *et al.*, 1985; Wang *et al.*, 1992; Goumans *et al.*, 1999). The mechanisms that form the cavity in a developing embryo and those that form the cavity in an EB are distinct however (Boyd *et al.*, 1984). In many differentiation systems, transcriptional activation and the subsequent expression of receptor molecules and cell surface markers to conduct signalling cascades for differentiation are identical to those seen in the tissue of question in the developing embryo.

For the purposes of this project, *in vitro* models of haematopoiesis and vasculogenesis in particular will be discussed in Section 1.9.

1.5. The isolation of Human ES Cells

Following the isolation of primate ES cells in 1995 (Thomson *et al.*, 1995), human ES cells were successfully isolated from blastocyst stage embryos for the first time in 1998 (Thomson *et al.*, 1998). Although initially they required growth on murine embryonic fibroblasts, a conditioning regime that eliminated the need for an actual feeder layer was eventually created (Xu *et al.*, 2001). These hES cells (including clonally derived cell lines) were found to be karyotypically normal, had high telomerase activity and were proven to be able to differentiate to the three germ layer both *in vivo* after injection into SCID mice (Thomson *et al.*, 1998) and *in vitro* following the generation of EBs(Itskovitz-Eldor *et al.*, 2000) (Amit *et al.*, 2000; Eiges *et al.*, 2001).

1.5.1.1. Differentiation Capability of HES Cells

The pattern of differentiation for hES cells is similar to what is seen for mES cells (as discussed in greater detail in section 1.4). Initially spherical structures, consisting of densely packed cells form. These eventually cavitate and form cystic EBs. Cells from all three germ layers have been detected including haematopoietic cells (Kaufman *et al.*, 2001), insulin secreting β cells (Assady *et al.*, 2001),

neuroectodermal cells (Reubinoff *et al.*, 2000; Schuldiner *et al.*, 2001) and cardiomyocytes (Itskovitz-Eldor *et al.*, 2000).

Endothelial cells have also been successfully isolated from hES cells (Levenberg *et al.*, 2002). 13-15 day-old EBs were found to contain vascular cells isolated on the basis of expression of platelet-endothelial cell adhesion molecule 1 (PECAM-1) and vascular endothelial cadherin (VE-Cadherin). Cells expressing these two markers formed vessel-like structures. When injected into SCID mice, PECAM-1^+ cells formed microvessels, some of which were found to exhibit functionality and had murine blood in their lumen. Interestingly, *flk-1* was expressed in undifferentiated cells. This was thought to represent either a differentiated subpopulation within the growing cells or was another variable between hES cells and mES cells that are reported not to express *flk-1* in the undifferentiated state.

1.5.1.2. Variation between hES and mES cells

There are several key differences between murine and human ES cells. The most obvious is the morphological variation that is apparent as well as hES cells' requirement for a more substantial matrix to attach to, either as a feeder layer or Matrigel. Whereas mES cells grow three-dimensionally as well as spreading over the culture surface, hES cells have been observed to grow flatter in culture. HES cells also appear to have a different surface marker expression pattern. Whereas mES cells show expression of stage-specific embryonic antigen-1 (SSEA-1) and lack of expression of SSEA-3 and SSEA-4, the opposite is seen in hES cells. Another interesting disparity between mES and hES cells is the inability of hES cells to respond to LIF (Thomson *et al.*, 1998; Pera *et al.*, 2000). This is also seen in human EC cells (Pera *et al.*, 1989; Roach *et al.*, 1993) and primate ES cells (Thomson and Marshall, 1998). In the murine embryo, LIF signalling is known to play a role in the survival of the epiblast through diapause (Nichols *et al.*, 2001); human embryos are probably unresponsive to LIF due to the absence of a mechanism for implantation delay. With the discovery that *nanog* signalling maintains pluripotency in the absence of STAT3 activation, evidence is suggesting that in hES cells, an alternative

mechanism for the maintenance of pluripotency is dominant over that initiated by STAT3 activation.

1.6. Adult Stem Cells as Alternative Sources of Therapeutic Cells

Stem cells persist in the adult, residing in niches that control their activity and proliferation. These adult stem cells (ASCs) enable lifelong cellular renewal in anatomical systems that require continual regeneration such as the haematopoietic, nervous and gastrointestinal systems and in the skin, hair follicle and the circulation (Raff, 2003).

The existence of ASCs was known of several decades before the discovery of ES cells, mainly through the study of the haematopoietic stem cell (Kumaravelu *et al.*, 2002). Their study has increased understanding of development and differentiation of various cell types and has led to transplantation therapies such as bone marrow transplantation for the treatment of blood disorders and leukaemias. The importance of the existence and function of a stem cell niche is highlighted by the lack of success when bone marrow transplantation is used for conditions such as myeloid metaplasia and myeloproliferative disorders where the stem cell niche is permanently damaged by fibrosis of the bone marrow. In such cases, when bone marrow transplantation is used, engraftment is often unsuccessful (Singhal *et al.*, 1995; Gurevitch *et al.*, 1999; Daley, 2002).

1.6.1. Traditional Beliefs Regarding ASC Potentiality

Until recently, it was believed that ES cells were the only truly pluripotent cell type; ASCs were thought to be multi-potent, with the ability to differentiate to form a set of distinct yet related cell types, but unable to differentiate to form cell types from a different tissue type or germ layer.

1.6.2. Possible Pluripotency of ASC

Several studies challenged these beliefs and hinted at the possible pluripotency of ASCs from a variety of niches. Not only do ASCs appear to be able to differentiate to lineages that are entirely unrelated to the location of their residing niche, but there have also been examples of stem cells differentiating into lineages that belong to another germ layer. First, it was shown that cells from muscle and the brain were able to contribute to haematopoiesis (Bjornson *et al.*, 1999; Jackson *et al.*, 1999). Haematopoietic stem cells, the best-characterised ASCs, have also been directed down many different lineages including hepatocytes and neurons (Lagasse *et al.*, 2000; Thiese *et al.*, 2000; Krause *et al.*, 2001). Muscle and blood cells have been derived from the stem cells of either system (Jackson *et al.*, 1999; Gussoni *et al.*, 1999) however, much controversy still remains regarding ASC plasticity. The possible mechanisms under play in ASC maintenance and plasticity include transdifferentiation, de-differentiation, fusion and the existence of multiple stem cell types or a universal pluripotent ASC (Wagers and Weissman, 2004).

One study by Catherine Verfaillie's group has identified a population of mesenchymal stem cells entitled multipotent adult progenitor cells (MAPCs) (Jiang *et al.*, 2002). These cells have shown remarkable potentiality and have been differentiated to numerous lineages from all three germ layers (Reyes and Verfaillie, 2001; Reyes *et al.*, 2002; Schwartz *et al.*, 2002; Zhao *et al.*, 2002; Zhao *et al.*, 2002). Upon injection, they are able to colonise the mouse blastocyst and form a chimaera, however they have yet to be shown to colonise the germline. MAPCs are a product of extended culture *in vitro*, under the stressful conditions of low density and low serum supplementation. It is a matter of some debate whether these cells actually reside in the bone marrow or whether culture of mesenchymal stem cells under these conditions results in the abrogation of the expected cell-cell interactions, signalling and growth factor release to maintain the normal potentiality of a mesenchymal stem cell. This loss of control in the absence of a niche could result in the cells being left to either die or differentiate and senesce, with a minority dedifferentiating to a more primitive and pluripotent state.

The Verfaille studies have been supported with the discovery of marrow-isolated adult multilineage (MIAMI) cells (D'Ippolito *et al.*, 2004). These cells are distinct from MAPCs with respect to their expression profile but have a similarly broad potentiality for differentiation. Although care was taken in order to recreate an *in vivo* microenvironment thought to support stem cells, these cells were also subject to culture at low density for a prolonged period of time and must be considered with caution as a true pluripotent stem cell population. Encouragingly, MIAMI cells did show *oct-4* and *rex-1* expression, both of which are typically expressed by ES cells. Also, MIAMI cells have been easily isolated from many individuals of varying age and do not appear to senesce or lose their differentiation potential following extensive time in culture (D'Ippolito *et al.*, 2004).

Knowledge of the effects of niches on the development of primitive progenitor populations, and the signalling pathways that control cellular migration, expansion and differentiation is accumulating fast. Learning more about the lineage boundaries in place and the resolution of several conflicting findings will hopefully not be long in the coming and will elucidate the true pluripotency of ASCs.

Recent investigations into the nuclear reprogramming capabilities of ES cells when cultured with somatic cells have given rise to an alternative explanation for ASC plasticity. Somatic neural cells and bone marrow cells were cultured with murine ES cells. Where the somatic cells were found to appear ES cell-like, it was found that the ES cells and the somatic cells had fused spontaneously, generating hybrid tetraploid cells that had the ability to proliferate rapidly and were able to contribute to the three germ layers (Pells *et al.*, 2002; Terada *et al.*, 2002; Ying *et al.*, 2002). Observations of spontaneous fusion *in vivo* have since been documented in the liver (Vassilopoulos and Russell, 2003; Wang *et al.*, 2003). Alvarez-Dolado *et al.* (2003) showed that bone marrow cells could contribute to non-haematopoietic lineages e.g. cardiomyocytes, hepatocytes and Purkinje neurons, but that in all instances, this contribution could be attributable to cell fusion. Only further understanding of the behaviour of ASCs and their tracking *in vivo* will enable their true mode of action and potentiality to be deciphered.

1.7. Ethical Implications of the Use of ES Cells

ES cells have key advantages for use as a therapeutic tool over ASCs. Their potential and molecular profile is better characterised and their differentiation to a multitude of lineages has to date been more easily achieved. They also proliferate more quickly than ASCs and can be maintained as healthy cell populations *in vitro* more easily. After their isolation from human embryos (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000), their potential use for generating cell-based therapies has been avidly investigated. ASCs have not been overlooked entirely for therapeutic applications however because they hold two key advantages over ES cells as a therapeutic tool. The ethical implications of isolating and utilising hES cells are already a key issue. Not only does their isolation require the destruction of a developing human embryo, but also, to obtain fully histocompatible cells would require therapeutic cloning of an embryo using a recipient-derived cell. Recipient-derived ASCs would be histocompatible, and the use of ASCs would overcome any moral issues arising from the destruction of a living embryo or carrying out the cloning process.

1.8. Gene Targeting

Germline modification was initially achieved by inserting exogenous DNA into the genome via retroviral transfection or direct pronuclear microinjection into a fertilised egg (Jaenisch, 1988). These methods of transgenesis lent themselves to the study of gene function and regulation. However, they were limited in their utility due to the crudeness of the techniques being used. DNA could only be inserted and the sites of integration were, on the whole, random.

The ability of endogenous chromosomal DNA and exogenous DNA to recombine at homologous sites has enabled the "targeting" and modification of any genetic locus. This strategy has been used for over a decade to introduce subtle changes to the mouse genome.

ES cell technology, specifically the ability to genetically modify ES cells using homologous recombination, has bridged genetic manipulation of the genome with the study of development in the mouse model. The development of gene-targeting has enabled scientists to make predetermined changes to most genetic loci and study the effects of the resulting genetic disruption in heterozygotes, and homozygotes where homozygosity is not lethal., Typically, a reporter gene is inserted into the locus of interest in an ES cell line, disrupting the expression of the endogenous gene whilst also sequestering the gene of interest's transcriptional control. Blastocyst injection of the modified ES cells can give rise to chimaeric mice with possible ES cell-derived germ cells. Backcrossing the chimaeras can generate heterozygotes, which in turn can be bred to homozygosity if the loss of gene function is not lethal. By tracking the reporter gene expression in the heterozygous and homozygous mutants, the expression pattern and gene function can be studies.

The first endogenous gene to be modified by gene targeting was the β-globin gene in foetal fibroblasts (Smithies *et al.*, 1985). The rate of homologous recombination to non-homologous recombination was 1:1000. The first instance of gene targeting in ES cells was carried out by Thomas *et al.* (Thomas *et al.*, 1986) where a similar targeting frequency was observed to that seen in foetal fibroblasts. This inherent ability provided a convenient method of modifying any genetic locus and introducing the resultant changes to the germline of the resulting chimaeric mice because karyotypically normal ES cells have the ability to colonise the germline (Bradley *et al.*, 1984).

1.9. *In Vitro* Models of Haematopoiesis

1.9.1. Spontaneous Haematopoietic and Vascular Differentiation

Although direct studies of embryogenesis have contributed to a greater understanding of early developmental processes such as haematopoiesis, technical difficulties have hampered discoveries. Such problems include accessing the embryo, which has a very low total cell number when the haematopoietic system

arises, distinguishing cell types e.g. identifying the haemangioblast from multipotent mesoderm and deciphering the potentiality of a progenitor population. *In vitro* differentiation of ES cells has enabled scientists to model the *in vivo* processes that take place for the development and differentiation of the haematopoietic system. The use of ES cells targeted at genetic loci believed to have critical roles in developmental processes have also aided the unravelling of the molecular mechanisms that guide differentiation and development.

When placed in conditions that promote the aggregation and formation of EBs from ES cells, the aggregates are able to differentiate spontaneously to many lineages (Evans and Kaufman, 1981; Martin, 1981; Doetschman *et al.*, 1985). EBs have been established in culture as a way of mimicking the spatial organization of differentiating cells in the embryo (Nishikawa *et al.*, 1998a). It was through these structures that haematopoiesis was first observed *in vitro* (Doetschman *et al.*, 1985; Wiles and Keller, 1991).

Doetschman *et al.* (Doetschman *et al.*, 1985) investigated the *in vitro* differentiation of EBs extensively. They noted that by day 8-10, approximately 50% of EBs were cystic. 30% of these exhibited cardiogenesis and when cultured in Iscove's modified Dulbecco Medium (IMDM) with cord blood serum 30% formed blood islands (Doetschman *et al.*, 1985). Cystic EBs have also been observed to form vascular channels from these blood islands, some of which were found to contain haematopoietic cells (Wang *et al.*, 1992). ES cell-derived haematopoietic cells showed the full complement of globin genes expressed in the correct temporal order as in the developing embryo (Lindenbaum and Grosveld, 1990). Plating in methylcellulose simulated the same colony forming cells as are derived from bone marrow (Schmitt *et al.*, 1991) and the synthesis of embryonic and adult globins (Wiles and Keller, 1991). The addition of erythropoietin increased the incidence of erythroid cells in methylcellulose cultures of ES cells (Wiles and Keller, 1991).

Close examination showed that the pockets of blood cells seen in the EBs were surrounded by an endothelial layer on either side (Doetschman *et al.*, 1985). This

observation of simple spontaneously differentiating EBs shows the close relationship of the vascular and haematopoietic lineages even in the absence of organising signals in an otherwise disorganised mass of differentiating cells. This ability has enabled the *in vitro* study of the molecular events that determine cellular differentiation in ES cells and can be extrapolated to probable events in the developing embryo. Until the development of the methylcellulose assay for haematopoietic colonies, only erythroid cells could be identified from differentiated EBs and their appearance was highly variable (Doetschman *et al.*, 1985). Definitive haematopoietic precursors can also be observed in EBs soon after primitive erythroid cells appear (Keller *et al.*, 1993).

1.9.2. Isolation of the Haemangioblast *in Vitro*

The haemangioblast is believed to emerge directly after mesoderm specification *in vivo* (*in vivo* development of the vasculature is discussed in greater detail in section 1.10). At this time, there are few cells in the developing embryo, which is, in turn, difficult to access for isolation of the haemangioblast. Studies of haemangioblast specification and characterisation have therefore turned to *in vitro* models from ES cell differentiation.

The BLast-Colony-Forming Cell (BL-CFC) is detected in ES cell culture and its existence goes some way to corroborating the possible existence of the haemangioblast. BL-CFCs respond to VEGF (see Section 1.10.4.1 for details of VEGF's action in haemangioblast specification), form blast colonies (undefined colonies in methylcellulose culture) that express FLK-1, a key marker of the putative haemangioblast (Section 1.10.4.2) and eventually differentiate down both haematopoietic and endothelial lineages after being grown in a methylcellulose matrix (Kennedy *et al.*, 1997; Choi *et al.*, 1998). In this culture system, both primitive and definitive haematopoietic elements are seen. The clonal nature of these colonies was confirmed with a mixing experiment whereby two differentially labelled ES cell lines were mixed prior to the setting up of the BL-CFC assay. It was found that the blast colonies were from either one or the other ES cell line (Kennedy *et al.*, 1997; Choi *et al.*, 1998).

BL-CFCs are evident in 2.5-3.5-day-old EBs (Kennedy *et al.*, 1997; Choi *et al.*, 1998). They are a transient population that appear before other haematopoietic and endothelial populations (Kennedy *et al.*, 1997; Choi *et al.*, 1998). In methylcellulose culture they form blast colonies that express FLK-1, SCL/TAL-1, CD34 and GATA-1, all of which are markers expressed by haematopoietic progenitors. The FLK-1/VEGF interaction is believed to be important for the proliferation and differentiation of these precursors. Residual haematopoiesis in *flk-1*$^{-/-}$ embryos show that the interaction is not required for mesoderm commitment as is true in the embryo (Kennedy *et al.*, 1997). It has been observed that when FLK-1^{+} cells are supplied with VEGF, both endothelial and haematopoietic cells form, however, when VEGF is not supplied, FLK-1^{+} cells differentiate down the haematopoietic lineage only (Eichmann *et al.*, 1997). This may appear to contradict findings that in the absence of VEGF, FLK-1^{+} cells differentiate to form mural cells (Yamashita *et al.*, 2000). However, as neither study addresses the possibility of other lineages being differentiated other than the ones of interest, it may be the case that both haematopoietic and smooth muscle cells form from FLK-1^{+} cells in the absence of VEGF.

BL-CFC did not express *brachyury* therefore they were at a more advanced stage than mesoderm (Kennedy *et al.*, 1997). They could be differentiated further to form haematopoietic colonies, expressed haematopoietic markers and both primitive and definitive globin genes. Blast colonies formed from BL-CFCs, expressed FLK-1 and responded to VEGF. They had the capacity to differentiate to endothelial and haematopoietic lineages. Once picked and plated in Matrigel, 30-40% of blast colonies derived from 3.25-day-old EBs could differentiate to both endothelial and haematopoietic lineages (Kennedy *et al.*, 1997). The remainder produced only haematopoietic progeny. The fact that blast colonies derived from so-called BL-CFCs appear to have the bipotentiality to differentiate down both endothelial and haematopoietic lineages supports the argument in favour of the existence of the haemangioblast (Choi *et al.*, 1998).

2.5-day-old EBs were found to have the highest (75%) level of bipotentiality and as the age of the EBs increased, the level of bipotentiality dropped. 3.5-day-old EBs had a <25% bipotentiality. This indicated that the BL-CFC was a transient cell population and a likely *in vitro* model of the presumptive haemangioblast.

Doetchmann *et al.* (Doetschman *et al.*, 1985) suggested that blood islands could only be induced via three-dimensional EBs, a concept that has been carried through by convention to the present day in the *in vitro* culture systems that have been developed. Nishikawa *et al.* (1998a) have done studies using two-dimensional cell differentiation that suggest a possible alternative to EB differentiation. Their culture system is based on using a collagen IV surface for cell growth and the reculturing of cells sorted at intermediate stages for common haematopoietic and endothelial cell surface markers. It is interesting that such differing culture systems could be utilised in similar studies in development. However it does present questions as to their reliability, based on the fact that the microenvironment that the cells are cultured in varies so much.

Nishikawa *et al.* (Nishikawa *et al.*, 1998b) generated lymphohaematopoietic cells from endothelial cells sorted from embryos on the basis if their expression of VE-Cadherin from 9.5 dpc embryos or YS. The endothelial cells were identified by their co-expression of FLK-1, CD31, and CD34 with VE-Cadherin. They also showed that endothelial cells that had already formed vessel structures had haematopoietic capabilities *in vitro*. It is however, unknown whether this capability is present *in vivo* and what mechanisms control haematopoietic cell differentiation if endothelia do have haemogenic capabilities.

Both the Keller and Nishikawa differentiation protocols are discussed further in Chapter 4.

1.10. The Vertebrate Haematopoietic System and its Development

1.10.1. Mechanisms Involved in the Formation of the Vasculature

The haematopoietic system and the organs involved directly in its generation and maintenance are the first to develop in ontogeny. Although intensely studied for over a century and despite there being a wealth of information on its development, there remains a great deal of ambiguity and uncertainty about the origin, progression and maintenance of the haematopoietic system.

The cell proposed for use in this project is believed to be the progenitor to all lineages involved in vasculogenesis. To understand its origin and subsequent role in development, the origins of haematopoietic development must first be reviewed.

Vasculogenesis gives rise to a homogenous lattice of blood vessels termed the primary capillary plexus from the differentiation, expansion and coalescence of vascular endothelial cells. The process of angiogenesis remodels this network and includes the sprouting of new and differentially sized blood vessels from the pre-existing ones established in vasculogenesis. Angiogenesis also involves the recruitment of associated tissues to the vasculature including supporting smooth muscle and pericytes (Folkman and D'Amore, 1996). Angiogenesis can occur where needed in the embryo and the adult: in cases of genuine tissue ischaemia and in pathological cases of neovascularisation such as tumorigenesis.

Neovascularisation does not rely solely on angiogenesis as evidence is emerging that indicates that vasculogenesis may also occur in wound healing, tumour vascularisation and tissue ischaemia. It has been shown that even after vascular formation, circulating endothelial progenitors can incorporate into vessel endothelium indicating the dynamism of the vasculature (Asahara *et al.*, 1999).

Pathological angiogenesis e.g. tumour vascularisation, in wounding, chronic inflammation and atherosclerosis is believed to proceed using the same mechanisms

employed in embryonic angiogenesis. Tumours are believed to release angiogenic growth factors to engage the angiogenic machinery and recruit the cells required to install a link to the existing vasculature.

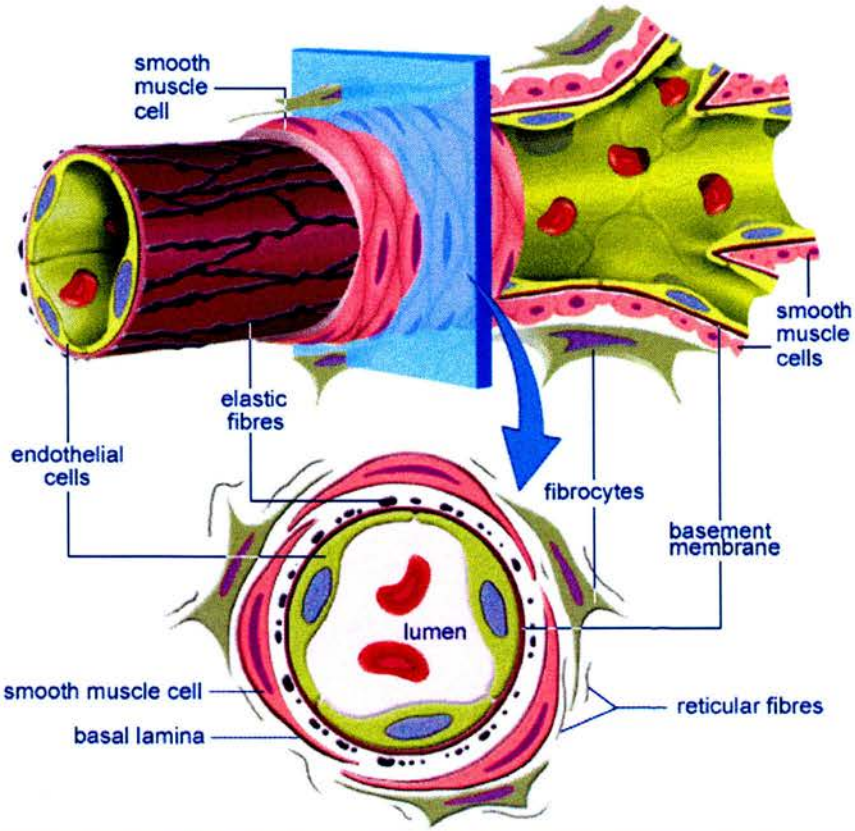

Figure 1.2 *Diagram showing the many components that form blood vessels. Adapted from http://www.mmi.mcgill.ca/mmimediasampler2002/beforeandafter/after.htm*

1.10.2. Initial Concepts on the Origins of the Haematopoietic System in the Murine System

Studies on the origins and developmental path of the mammalian haematopoietic system have been carried out for over a century. This has led to the accumulation of a wealth of information on vasculogenesis and haematopoiesis. Vera Danchakoff (Danchakoff, 1916) and Florence Sabin (Sabin, 1920) made insightful observations of chick haematopoiesis that laid the foundations for existing models of haematopoietic development. However, despite this, the current models for haematopoietic development are still very fluid and constantly developing.

The mesoderm forms at 6 dpc from signalling events between the primitive endoderm and ectoderm (Dzierzak *et al.*, 1998). Mesoderm migrates through the primitive streak contributing to the visceral yolk sac (YS) and other structures (Haar and Ackerman, 1971; Silver and Palis, 1997). The onset of haematopoiesis occurs at the primitive streak stage: 7 dpc (**E7, Figure 1.3**).

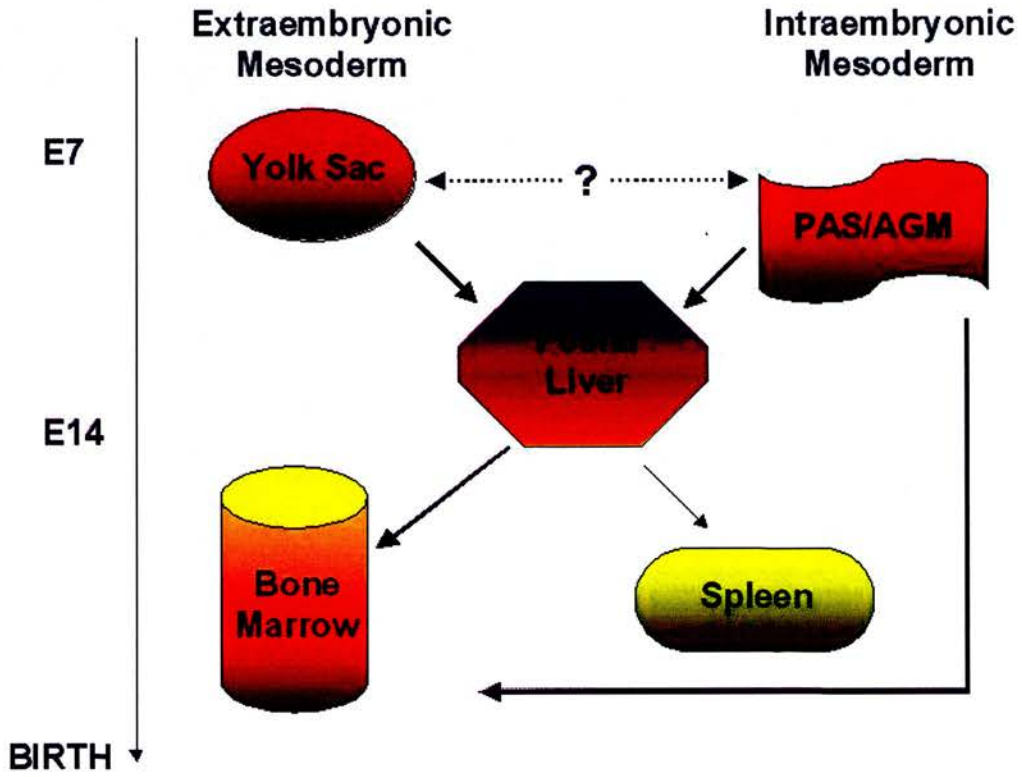

Figure 1.3 *Schematic representation of the course of haematopoietic colonisation through murine ontogeny (stages shown on left). Haematopoiesis is first observed in the extraembryonic YS at 7-7.5 doc. The para-aortic splanchnopleura/ aortic-gonad mesonephros (PAS/AGM) region is next colonised although whether the haematopoietic progenitors arise independently or migrate from the YS is still controversial. The foetal liver is next colonised followed by the spleen and the bone marrow at birth.*

Haematopoiesis begins in the embryonic YS with the formation of blood islands (Danchakoff, 1916; Moore and Owen, 1967). Blood islands appear from aggregates of mesoderm in the YS (Moore and Metcalf, 1970; Yamaguchi *et al.*, 1993; Shalaby *et al.*, 1997). The peripheral flat cells of these blood islands differentiate to form angioblasts i.e. endothelial precursors, while the inner round cells form primitive erythrocytes (Shalaby *et al.*, 1997; Dzierzak *et al.*, 1998; Robb and Elefanty, 1998)

(**Figure 1.4**). Primitive but committed haematopoietic progenitors were detected in the YS between 7 and 8.5 dpc. These cells were nucleated and expressed foetal globin isoforms (Moore and Metcalf, 1970; Johnson and Barker, 1985; Wong *et al.*, 1986). These observations of extraembryonic haematopoiesis have set a paradigm of embryonic haematopoiesis arising in the YS followed by the colonisation of the embryo by YS-derived haematopoietic progenitors. As development advanced, a multipotent stem cell population derived from differentiation in the YS was believed to migrate to the foetal liver via the established circulatory system, then to the foetal spleen and finally the adult bone marrow (**Figure 1.3**). Initially, YS-derived haematopoietic cells were believed to repopulate the entire haematopoietic system, and parallels were drawn between the avian and the mammalian system (Moore and Metcalf, 1970). YS stem cells were also thought to be capable of repopulating the thymus and the lymphoid system adding support to this theory of multipotency, as at 8 dpc, the YS was found to contain erythroid and some lymphoid progenitors. The embryo proper did not contain such cells and at 7.5 dpc, the embryo was believed to be unable to develop any cells of the myeloid lineage in the absence of the YS (Moore and Metcalf, 1970).

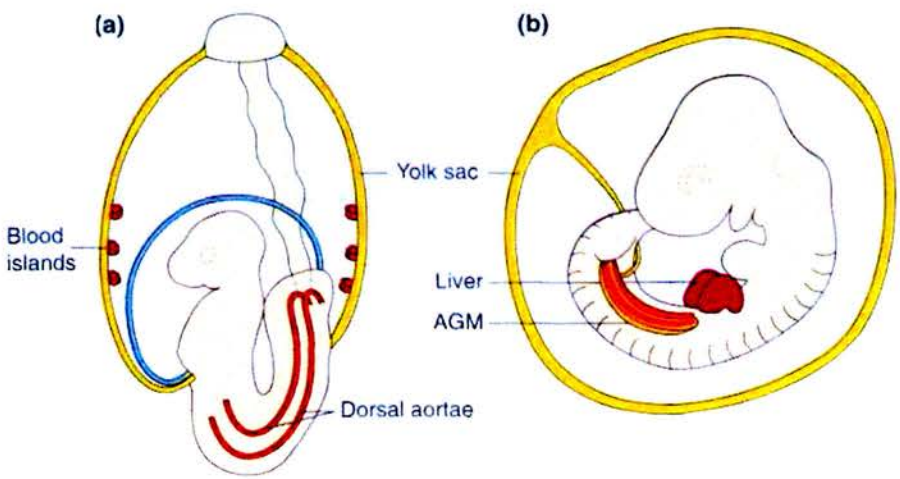

Figure 1.4 *Regions annotated mark the sites of haematopoiesis in the murine embryo at: (a) 8.5 dpc, (b) 10.5 dpc (Dzierzak et al., 1998).*

This understanding of mammalian haematopoiesis was challenged by the theory that haematopoiesis arises at several sites in the developing mammalian embryo including the YS.

Avian studies were central to the development of the theory that haematopoietic cells initially developed in the YS (Dieterlen-Lievre, 1975); Dieterlen-Lièvre and colleagues extended their investigations by generating chick-quail YS chimaeras where quail embryos were grafted onto chick YS to look at the origins of the most primitive haematopoietic progenitors. Initial studies revealed that intraembryonic haematopoiesis succeeded YS haematopoiesis (Dieterlen-Lievre *et al.*, 1976) and had the potentiality to colonise the YS (Martin *et al.*, 1978a) as well as other intraembryonic sites of haematopoietic development (Dieterlen-Lièvre, 1975).

1.10.3. Developing Controversies on the Origins of the Haematopoietic System

Grafting experiments challenged the initial concepts of YS haematopoiesis being the origin of all subsequent haematopoiesis as they provided evidence suggesting that definitive haematopoiesis originated independently in the intraembryonic aorta gonad mesonephros (AGM) region and not the YS (Dieterlen-Lièvre, 1975; Beaupain *et al.*, 1979; Lassila *et al.*, 1982). The amphibian system had been observed to have two distinct sites of haematopoietic activity as well (Turpen *et al.*, 1981). Support for this theory also came from observations that lymphoid and myeloid precursors existed in the para-aortic splanchnopleura (PAS) (which develops to form the AGM as early as 7.5 dpc in mice, a time when circulation has not been established (Shalaby *et al.*, 1997). The PAS/AGM comprises the endoderm of the developing gut, the endothelium of blood vessels (dorsal aorta and omphalomesenteric artery), the gential ridges, the pro/mesonephros and the splanchnic mesoderm enveloping them.

Erythrocytes are first visible at 7.5 dpc in the YS. 8-8.5 dpc YS can be made to generate B and T lymphocytes showing an inherent potentiality for definitive haematopoiesis if given the right cues (Palacios and Imhof, 1993). By 9 dpc, YS haematopoietic cells have repopulating potential (Dzierzak *et al.*, 1998) with long-term repopulating haematopoietic stem cells (LTR-HSCs) seen at 11 dpc (Moore and Metcalf, 1970; Medvinsky *et al.*, 1996; Shalaby *et al.*, 1997; Dzierzak *et al.*, 1998).

However, at this stage, the circulatory system has been established, therefore LTR-HSCs could have seeded the YS from the embryo (Shalaby *et al.*, 1997). The focus of investigation turned to the PAS/AGM. Parallel haematopoietic activity was observed in the PAS/AGM region by 8 dpc, a time when the circulatory system has yet to link YS haematopoiesis to the embryo proper (Cumano *et al.*, 1993; Huang and Auerbach, 1993; Godin *et al.*, 1993).

The colony-forming units-spleen (CFU-S) assay involves tail vein injections of the haematopoietic progenitors being assessed for their capability to form multilineage haematopoietic progenitors *in vivo* by their ability to colonise the spleen of lethally irradiated mice and form colonies (Till and McCulloch, 1961). Embryonic tissue is isolated, grown in culture, dispersed to a single-cell suspension and injected into lethally irradiated mice where potent haematopoietic progenitors are found to colonise the spleens of these mice with a frequency that is in direct relationship to their estimated haematopoietic potential. This assay indicates that despite the simultaneous appearance of YS and AGM haematopoietic cells, those originating in the AGM are more potent (Medvinsky *et al.*, 1996; Dzierzak *et al.*, 1998). At 8.5 dpc in the mouse, multipotent haematopoietic cells were found that were able to form (Godin *et al.*, 1995). CFU-S were detectable in both the YS and embryo at late day 9 when the circulation has been established (Medvinsky *et al.*, 1993). 10 dpc AGM CFU-S are similar in potentiality and composition to 11 dpc liver CFU-S therefore there may be a migratory or co-developmental relationship between the two structures. AGM-derived CFU-S were also morphologically more similar to foetal liver CFU-S than YS-derived CFU-S (Medvinsky *et al.*, 1996). *In vitro* assays show that YS cells do not have multipotent potential before circulation has been established, only erythroid and myeloid potentials (Dzierzak *et al.*, 1998). The model that evolved after these convincing investigations was that haematopoiesis occurred in two distinct waves: a "primitive" one in the YS where the haematopoietic cells that formed had little proliferative or repopulating potential, and a second wave of "definitive" haematopoiesis within the AGM (Dzierzak *et al.*, 1998; Palis *et al.*, 1999). This second wave was believed to give rise to HSC with long-term repopulating capabilities and it was believed that it was only after circulation was

established post-8.5 dpc that the YS acquired these haematopoietic cells and consequently the same LTR ability (Medvinsky *et al.*, 1996; Dzierzak *et al.*, 1998).

Despite the popularity of this model, the significance and LTR ability of the first YS precursors has remained an area of investigation. It has been argued that YS cells prior to 11 dpc failed to show LTR ability in lethally irradiated mice because they require a haematopoietic microenvironment to home to (Lu *et al.*, 1996; Medvinsky and Dzierzak, 1996; Delassus *et al.*, 1995). Haematopoietic cells found in the AGM are able to reconstitute an irradiated adult mouse following intravenous injection of the purified progenitors (Muller *et al.*, 1994; Medvinsky and Dzierzak, 1996). Age matched YS cells do not exhibit this ability, however they are able to completely reconstitute busulphan[1] conditioned newborn mice (Yoder *et al.*, 1997b; Yoder *et al.*, 1997a). These disparate findings can be reconciled if it is accepted that the newborn mice may provide a microenvironment which is permissive for proliferation and the development of the full spectrum of cell fates that the YS precursors can home to. In neonatal mice, haematopoietic activity is present in the liver, spleen and bone marrow (Wolf *et al.*, 1995). It has been hypothesised that YS cells may graft in neonatal mice that have active haematopoiesis but not in adult mice where liver haematopoiesis no longer takes place (Yoder *et al.*, 1997a; Yoder *et al.*, 1997b). It may hint that YS haematopoietic precursors have the full LTR potentiality seen in the progenitors of the AGM but that until circulation is established, the YS microenvironment limits their differentiation until they migrate to the AGM where they differentiate further, proliferate and are directed to colonise the haematopoietic organs of the embryo, namely the foetal liver, spleen, thymus and eventually the bone marrow. The CFU-S generating progenitors are immature and highly proliferative. These progenitors represent the haematopoietic cells from 10 dpc onwards as haematopoietic cells from earlier stages of haematopoietic development do not produce CFU-S. The assay assesses cells with LTR *ability* and not LTR *potential*. This assay therefore cannot accurately assess the potentiality of the YS haematopoietic precursors that arise in the YS or AGM before 10 dpc because they

[1] Busulfan is an alkylating agent that interferes with DNA replication and is used in myelosuppression.

have not been primed for colonisation of other organs, such as the spleen in the CFU-S assay.

When cultured on AGM-derived stromal cells however, YS derived from 8.5 day old embryos were found to be able to reconstitute adult lethally irradiated mice (Matsuoka *et al.*, 2001) indicating that the AGM microenvironment may have an inductive effect on haematopoietic progenitors allowing for their acquisition of LTR activity. A proposal that YS-derived progenitors have properties that impede their survival in the adult mouse has been put forward after more recent findings by Cai *et al.* (Cai *et al.*, 2000) who showed that YS from *runx1* heterozygous mice were able to reconstitute the haematopoietic system of adult irradiated mice. The YS cells used were from the 10-dpc embryos when the circulation would have been established so the findings are not conclusive.

Further investigations in *Xenopus* by Turpen *et al.* (Turpen *et al.*, 1997) showed that by excising the two regions responsible for embryonic (ventral blood island) and adult haematopoiesis (dorsal lateral plate) and substituting one for the other, that both regions had the nascent ability to produce adult and embryonic blood and that it was the microenvironmental signals that determined the type of haematopoiesis that took place.

1.10.4. Key Elements in Vasculogenesis

To understand the sequence of events and molecular interplay involved in the specification and differentiation of mesoderm, the spatial and temporal expression of key receptors, ligands and transcription factors have been gradually dissected.

1.10.4.1. Vascular Endothelial Growth Factor

The VEGFs are essential for haematopoietic development during ontogeny, postnatally and in the adult in instances of severe wounding or ischaemia. VEGF/VEGF-A/Vascular Permeability Factor is a heparin-binding glycoprotein that is secreted as a homodimer (Houck *et al.*, 1991; Houck *et al.*, 1992; Park *et al.*, 1993) VEGF can increase vascular permeability, cause vasodilatation following activation

of nitric oxide synthase, stimulate migration and inhibit apoptosis. It is a key mitogen in the activation and differentiation of the vascular lineages. Although endothelial cells respond to it, they rarely secrete it. It has also been found to be a regulator of tumour angiogenesis (Millauer *et al.*, 1994).

There are several splice variants for VEGF 121, 165, 189 and 206. The last is only commonly found in the foetal liver . More recently 145 and 183 amino acid variants have also been detected (Kozlowska *et al.*, 1998; Liu *et al.*, 1999). 165, 189 and 206 have heparin-binding domains that anchor them to the extracellular matrix and bind with heparin sulphate to aid presentation of VEGF to its receptors.

VEGF-A expression is responsive to hypoxia, glucose concentration, pH as well as the activity of certain oncogenes. Any element that induces the activation of mitogen-activated protein kinase (MAPK) activates VEGF simultaneously (Milanini-Mongiat *et al.*, 2002) e.g. phosphorylation of hypoxia-inducible transcription factor 1α (HIF-1α) by MAPKs enhances VEGF transcription (Hofer *et al.*, 2001). **Figure 1.5** shows the various downstream effectors of VEGF signalling. As can be seen, the precise mechanisms for the various actions are still being formulated.

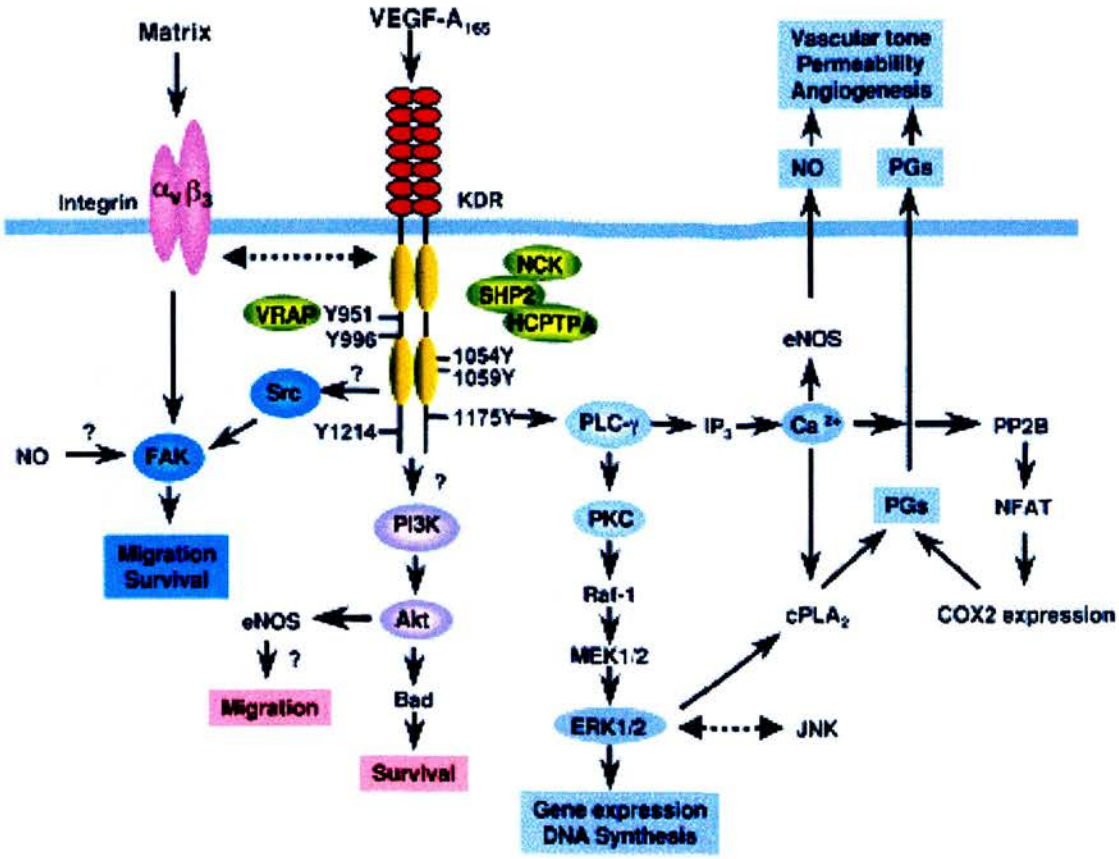

Figure 1.5 *Signalling activity following KDR (VEGFR2/FLK-1) by VEGF. Activation of KDR/FLK-1 results in receptor dimerisation and autophosphorylation at numerous tyrosine residues of the cytoplasmic domain of the receptor. Various downstream signalling mechanisms can be activated resulting in a variety of responses including migration, cell survival, regulation of vascular permeability and the activation and regulation of angiogenesis and vasculogenesis. Figure taken from Zachary, 2003.*

VEGF B, C, D and placental growth factor (PlGF) (Schreiber *et al.*, 2000) are also part of the VEGF ligand family and related to the platelet-derived growth factor (PDGF) A and B molecules. **Figure 1.6** shows the binding of these ligands to their respective receptors. These ligands are less well characterised than VEGF though it is known that there is little functional overlap between them despite the nomenclature: VEGF-B (Grimmond *et al.*, 1996), VEGF-C (Joukov *et al.*, 1996; Lee *et al.*, 1996), VEGF-D (Orlandini *et al.*, 1996; Yamada *et al.*, 1997; Achen *et al.*, 1998). Generally, they are all expressed by mesenchymal and epithelial cells and have paracrine influences on adjacent cells expressing the relevant receptors.

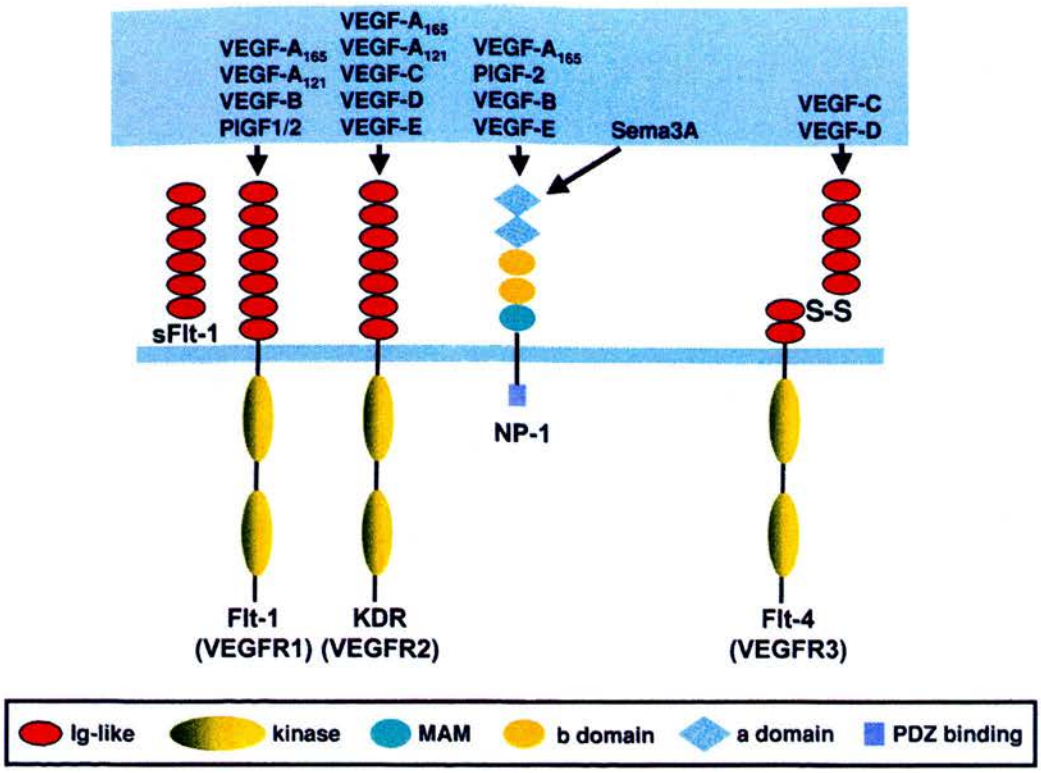

Figure 1.6 *The VEGF receptor family and their ligands. There are three key receptors, each of which can bind a range of VEGF molecules. VEGF receptors comprise an immunoglobulin-like extracellular domain and a cytoplasmic domain comprising a kinase domain. Neuropilin-1 (NP-1) is a non-tyrosine kinase receptor for the VEGF family of ligands and consists of an extracellular domain with mephrin, AS, μ tyrosine phosphatase (MAM). Figure taken from Zachary, 2003.*

All VEGFs bind to one of the VEGF receptors and activate their intracellular tyrosine kinase domain (**Figure 1.6**). Three receptors commonly respond to VEGF stimulation: R1/FLT-1, R2/FLK-1, and R3/FLT-4. Neuropilin-1 and 2 are also activated by VEGF binding. R3 is specific to lymphatic endothelium.

VEGF receptors have 7 extracellular immunoglobulin-like domains, a single transmembrane domain and an intracellular tyrosine kinase domain, which is responsible for signal transmission on VEGF binding (Eichmann *et al.*, 1997).

VEGF (VEGF-A) is expressed in numerous pathological disorders including retinopathies, psoriasis, tumours, vascular lesions and rheumatoid arthritis (Carmeliet and Jain, 2000; Ferrara and Alitalo, 1999). The importance of VEGF in tumorigenesis has been demonstrated with the use of dominant negative VEGF

receptors (Millauer *et al.*, 1996) and blocking antibodies against VEGF and VEGF-R2 (Witte *et al.*, 1998) all of which have successfully blocked angiogenesis and tumour growth. The VEGF signalling pathway is therefore a popular target for anti-tumour therapies (Laird *et al.*, 2002; Fernando and Hurwitz, 2003; Zhang *et al.*, 2003).

Knocking out the function of a single VEGF allele is sufficient for lethality between 10-12 dpc (Ferrara *et al.*, 1996; Carmeliet *et al.*, 1996). It has been speculated that the lethality is a result of insufficient FLK-1 activation (Eichmann *et al.*, 1998). This highlights the importance of gene dosage in its role in development. Embryos sustain abnormalities to the heart, and dorsal aortae, and have a reduced number of red blood cells in the YS resulting in tissue necrosis, presumably from the absence of a functional vasculature. This indicates that VEGF function is dose-dependent and a threshold for sufficient activity exists (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996).

VEGFR2 (FLK-1 in mice and KDR in humans) is the central receptor for VEGF to trigger vasculogenesis and vascular permeability (Gille *et al.*, 2001). VEGF is expressed by the visceral endoderm of extra-embryonic endoderm when blood islands first emerge and in intra-embryonic endoderm as well. Initially some expression is also detected in the mesoderm. Cells expressing the FLK-1 receptor respond to VEGF in a paracrine manner. FLK-1 is also upregulated by VEGF (Kremer *et al.*, 1997). FLK-1 is the dominantly required receptor for VEGF during vasculogenesis, however FLT-1 appears to be more necessary for angiogenesis and the vascular remodelling that takes place as part of the process.

Formation of mature vessels relies on subtle interplay between the VEGFs, the angiopoietins that have a stabilising effect on vessel formation, and PDGFs. The expression of VEGFs leads directly to endothelial proliferation and tubulogenesis. The surrounding tissues of the formed, but immature blood vessels next express the angiopoietins that lead to the induction of PDGFs that induce the migration of pericytes and smooth muscle cells to support the vessels.

The action of VEGF alone is unable to direct the correct formation and organisation of blood vessels. A transgenic approach to targeted VEGF expression in the lens of the mouse eye resulted in the proliferation of endothelial progenitors at the site of expression but did not result in organised blood vessel formation (Ash and Overbeek, 2000).

Recently, VEGF has been found to have a role in neuroprotection. Insufficient levels of VEGF have been implicated in the occurrence of the neurodegenerative disorder amyotrophic lateral sclerosis, although the exact mechanisms underlying its role have yet to be discovered (reviewed in Storkebaum *et al.*, 2004).

1.10.4.2. FLK-1 Receptor Tyrosine Kinase

The haemangioblast's existence has been postulated by scientists (despite it never having been isolated) because of the markers that are shared by both the endothelial and haematopoietic lineages. These markers include CD34, SCL/TAL1, TIE-1 and TIE-2 and c-KIT. However one particular receptor tyrosine kinase stands out as the strongest candidate as a marker of the haemangioblast: foetal liver kinase-1 (Yamaguchi *et al.*, 1993). FLK-1's importance is consistently emphasized in development, however its precise role remains somewhat ambiguous.

Receptor tyrosine kinases are a group of transmembrane receptors that have growth factors as their ligands (Millauer *et al.*, 1993). They are regulators of growth and differentiation. Upon binding, these receptors undergo phosphorylation, as do other cellular components, thus inducing a complex cellular response (Ullrich and Schlessinger, 1990). FLK-1's extracellular region contains seven immunoglobulin-like domains similar to other members of the RTK superfamily of membrane receptors such as *c-kit*, and *c-fms*. Although considerably larger, it exhibits a high level of homology with *flt-1* (de Vries *et al.*, 1992) initially isolated by Shibuya *et al.* (1990) and with mirrored domain distributions. The intracellular domain of *flk-1* showed a greater level of similarity with other RTKs with sites for tyrosine kinase autophosphorylation.

In mouse, chick, zebrafish and *Xenopus*, *flk-1* is one of the earliest genes expressed during haematopoiesis and vascularisation (Cleaver and Krieg, 1998; Dumont *et al.*, 1995; Eichmann *et al.*, 1993; Flamme *et al.*, 1995; Fouquet *et al.*, 1997; Thompson *et al.*, 1998; Yamaguchi *et al.*, 1993). FLK-1 has a very high affinity for VEGF and their interaction plays a crucial role in development, being essential for vasculogenesis and angiogenesis (Millauer *et al.*, 1993; Quinn *et al.*, 1993). VEGF's interactions with FLK-1 are essential to invoke VEGF's full potential of possible responses (Ferrara *et al.*, 1996).

FLK-1 expression occurs initially at 7 dpc in the YS as blood islands form. Its expression precedes that of VEGF's other main receptor involved in vasculogenesis, *flt-1* (de Vries *et al.*, 1992; Peters *et al.*, 1993). The timing of *flk-1* expression corresponds to the time when haemangioblasts are expected to arise. Soon after, *flk-1* expression is lost from cells in the haematopoietic lineage but is maintained in endothelial cells throughout ontogeny although expression decreases as foetal age increases. This corresponds to the decline in haematopoiesis and vasculogenesis as well (Kabrun *et al.*, 1997). Small colonies of adult HSCs maintain *flk-1* expression. Expression is also elevated at times of neovascularisation in the adult as well (Plate *et al.*, 1993). However, FLK-1 is not expressed on committed haematopoietic cells.

The *flk-1*$^{-/-}$ mutation is lethal to the embryo with a complete absence of organized blood vessels and necrosis (believed to be due to circulatory failure) by 8.5 dpc. *flk-1*$^{+/-}$ targeted ES cells that were subsequently used in aggregation chimeras showed that FLK-1^{+} cells derived from *flk-1*$^{+/-}$ heterozygous knockout ES cells were able to contribute to the endocardium, dorsal aorta, intersomitic vessels and the YS vasculature (Shalaby *et al.*, 1995). When the same study was carried out using *flk-1*$^{-/-}$ double knockout ES cells, no ES cell contribution to these lineages was seen. The wild-type embryo cells contributed to the haematopoietic cell fates while the *flk-1*$^{-/-}$ ES cells were directed to other fates (Shalaby *et al.*, 1997). This exhibited a cell autonomous requirement for *flk-1* expression in the vasculature. In knockout embryos, haematopoietic and endothelial precursors were thought to form normally

as was seen at the site of mesoderm differentiation, but they were believed to rely on the FLK-1-mediated signal for further differentiation or migration to an appropriate site of differentiation. It may be that VEGF signalling from the endoderm of the YS attracts FLK-1^+ cells to the YS and so in the absence of the signalling event, the mesoderm responds to other signals for alternative cell fates. The fact that *flk-*$1^{-/-}$ cells failed to form haematopoietic and endothelial cells supports the case that FLK-1 is essential to both processes and marks the putative haemangioblast (Shalaby *et al.*, 1997).

Analysis of *flk-*$1^{-/-}$ chimaeric embryos has revealed that ablation of the haematopoietic pathway may relate to a failure in a critical migratory step of FLK-1 deficient cells from the posterior primitive streak to the YS as well as possible intraembryonic sites of definitive haematopoiesis. This failure, established at 8.5 dpc, supports the theory that FLK-1's role in the embryo is to mark cells for migration to sites of endothelial and haematopoietic differentiation in response to other growth factors present there and not for differentiation itself (Schuh *et al.*, 1999). The fact that haematopoietic and endothelial precursors arise even in the absence of FLK-1 supports the view that FLK-1 is a marker that aids maintenance of these lineages and that earlier signals are responsible for the mesodermal differentiation, giving rise to the cells. *Flk-*$1^{-/-}$ ES cells retain the ability to form haematopoietic and endothelial cells in EBs (Schuh *et al.*, 1999; Hidaka *et al.*, 1999). This supports the hypothesis that *flk-1* is involved in migration to sites of proliferation and differentiation because you have removed the constraints of spatial niches or the same stringent regulation of cell migration and morphogen gradients that exist *in vivo*.

BL-CFCs that differentiate in *flk-*$1^{-/-}$ EBs form normally but are unresponsive to VEGF. Blast colony numbers are reduced, as are their sizes, however, there is no haematopoietic or endothelial development (Kabrun *et al.*, 1997; Schuh *et al.*, 1999). If this is similar to what occurs *in vivo*, then haemangioblasts are clearly not involved directly in differentiation.

When *flk-1*$^{-/-}$, $^{+/-}$ and $^{+/+}$ EBs were compared, their haematopoietic and endothelial differentiation potentials were similar. At 7.5 dpc *flk-1*$^{-/-}$ embryos have what is considered to be a normal number of haematopoietic progenitors and yet the 8.5 dpc embryo is highly deficient in such cells (Schuh *et al.*, 1999). This shows that FLK-1 signalling is crucial in the specification of cell fate with respect to haematopoiesis and vasculogenesis. However, it is not the differentiation-inducing factor itself; it is likely to be involved in ensuring the correct migration and expansion of all haematopoietic progenitors corroborating in vivo observations (Shalaby *et al.*, 1995; Shalaby *et al.*, 1997).

1.10.4.3. FLT-1 Receptor Tyrosine Kinase

FLT-1, VEGF's alternative receptor, primarily binds to a different isoform of VEGF to the one that FLK-1 recognises. *Flt-1*$^{-/-}$ embryos show an increase in endothelial cells with a corresponding vascular disorganization (Fong *et al.*, 1995; Fong *et al.*, 1999). The loss of organization appears to occur as a result of endothelial over-proliferation rather than as a separate and direct consequence of the knockout. If the kinase domain of FLT-1 is deleted, leaving the ligand binding domain as normal, homozygous null mice appear normal and develop no vascular abnormalities (Hiratsuka *et al.*, 1998). FLT-1 appears to act as a "VEGF sink" to regulate the amount of VEGF available for vascular development. In the absence of FLT-1 signalling, regulation ceases and endothelial proliferation results (Fong *et al.*, 1999). FLT-1 may even be responsible for the regulation of the differentiation of mesoderm to haemangioblastic cell fates.

If *flt-1* ablation results in haemangioblast proliferation due to regulatory failure, *flt-1* knockout animals would be ideal as a means of harvesting haemangioblast cells for transplantation, given their natural rarity at the very early stage of development when they are present. This relies on their isolation at an early enough stage to avoid the vascular disorganisation that would eventually ensue. High levels of VEGF in the system may successfully "swamp" the *flt-1* sink to simulate a situation similar to its absence although it cannot be ruled out that vascular disorganisation and other knock on implications of *flt-1* overexpression could prevent the utility of such a strategy. It

must be considered however, that due to several different isoforms of VEGF existing, each being recognised by a different selection of receptors, unpredictable outcomes may result from interference.

1.10.4.4. SCL/*tal I* Transcription Factor

SCL/TAL-1 was discovered at the breakpoint of a chromosomal translocation in cases of T-cell acute lymphoblastic leukaemia (T-ALL) placing the then unknown *scl/tal-1* gene beside the T cell receptor (Begley *et al.*, 1989; Finger *et al.*, 1989; Chen *et al.*, 1990). SCL/TAL-1 is a basic helix-loop-helix protein expressed in blood, endothelial cells and the brain. It is a transcription factor that controls the proliferation of multipotent haematopoietic cells as well as a positive regulator of erythroid differentiation; (Robb *et al.*, 1995; Shivdasani *et al.*, 1995; Robb *et al.*, 1996; Gering *et al.*, 1998).

SCL/TAL-1 has a role in primitive and definitive haematopoiesis (Porcher *et al.*, 1996) and is also expressed in the YS endothelium. It is expressed during a short developmental window from 7.5 dpc in the blood islands (Elefanty *et al.*, 1997; Robertson *et al.*, 2000; Endoh *et al.*, 2002). *Flk-1* expression has been shown to precede *scl/tal-1* in the developing embryo and is more widely expressed; however, the markers are believed to be associated (Minko *et al.*, 2003). SCL/TAL-1 expression mimics the expression patterns of FLK-1, except that *scl/tal-1* transcripts are not detected in the precardiac angioblasts that form the endocardium (Stainier *et al.*, 1993; Liao *et al.*, 1997). When appropriate expression is knocked out, both primitive and definitive haematopoiesis are blocked. In knockout studies lethality occurs early in gestation (8.5dpc) due to the failure of haematopoietic precursors to form in the YS (Shivdasani *et al.*, 1995; Robb *et al.*, 1995) (**Figure 1.7**). Both FLK-1 and SCL/TAL-1 expressing cells develop when the other gene has been knocked out. *Scl/tal-1*$^{-/-}$ ES cells can form endothelial cells (Faloon *et al.*, 2000; Robertson *et al.*, 2000) showing that the most likely markers of the haemangioblast are independently regulated and may play different roles in the establishment of the haematopoietic and vascular systems. These endothelial cells that form cannot form the primitive vascular plexus or undergo remodelling (Visvader *et al.*, 1998; Elefanty

et al., 1999) showing a requirement for SCL/TAL-1 in endothelial specification or the requirement for haematopoietic precursors at the earliest stages of vascular development.

Ectopic expression of *scl/tal-1* results in overproduction of FLK-1$^+$, SCL/TAL-1$^+$ cells that are possibly haemangioblastic (Gering *et al.*, 1998). This results in a greater proportion of FLK-1$^+$ cells being directed down the haematopoietic and haemogenic endothelial lineages (Endoh *et al.*, 2002). This imbalance in the distribution of differentiating FLK-1$^+$ cells results in fewer FLK-1$^+$ cells being directed to the smooth muscle lineage (Ema *et al.*, 2003). This may be because SCL/TAL-1 and MyoD - a master regulator for the muscle lineage, compete for common partners (e.g. E bHLH proteins) (Goldfarb and Lewandowska, 1995).

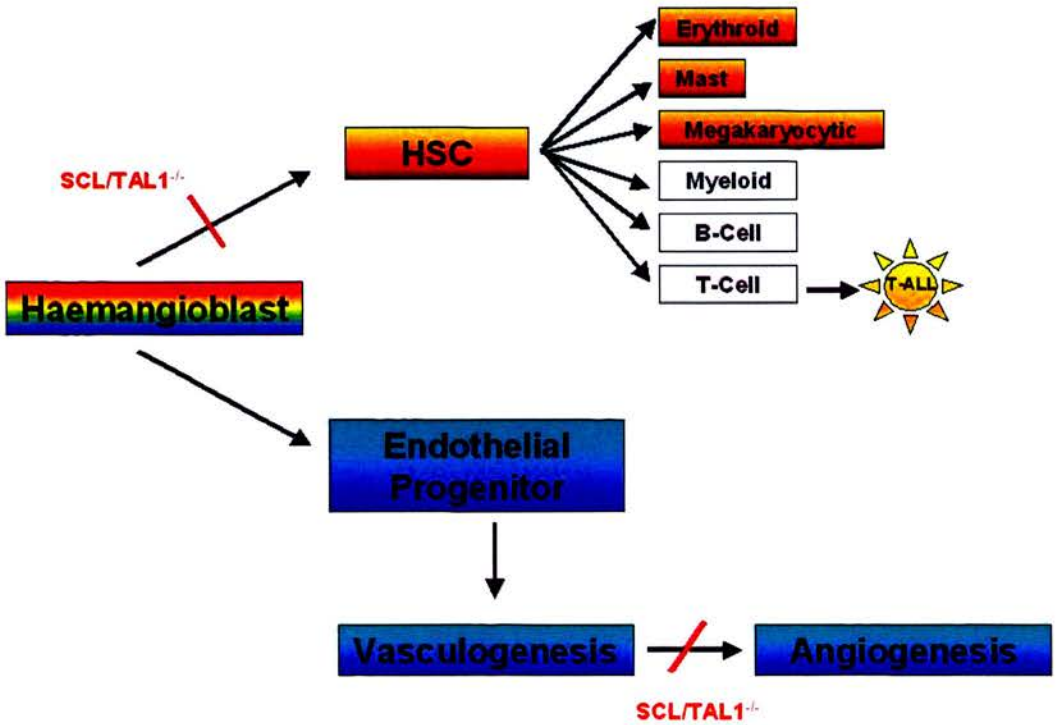

Figure 1.7 *Schematic of the expression pattern of SCL/TAL1findings of gene targeting experiments at the scl/tal-1 locus. Coloured boxes mark stages of haematopoietic and vascular development when SCL/TAL1 is expressed. The ✹ shows aberrant expression. The stages and effects of SCL/TAL1 knockout are shown with red lines. (Figure adapted from Barton et al., 1999).*

In SCL/TAL-1$^{-/-}$ mice, vasculogenesis proceeds normally (Robb *et al.*, 1995; Shivdasani *et al.*, 1995; Visvader *et al.*, 1998) and it is the absence of the haematopoietic lineage that is the direct cause of death. *Scl/tal-1*$^{-/-}$ ES cells can only differentiate down the endothelial lineage (Gering *et al.*, 1998; Faloon *et al.*, 2000; Robertson *et al.*, 2000), with *scl/tal-1*$^{-/-}$ EBs expressing FLK-1 and other endothelial cell markers at higher levels than in *scl/tal-1*$^{+/+}$ cells. In culture, a fraction of all FLK-1^{+} cells differentiate into BL-CFCs (the *in vitro*-derived putative haemangioblast; discussed in 1.9.2). All BL-CFCs are high expressers of SCL/TAL-1 as well as FLK-1 (Chung *et al.*, 2002). Chung *et al.* also showed that *flk-1* expression precedes *scl/tal-1* expression and that FLK-1^{+}, SCL/TAL-1^{+} cells form before FLK-1^{+}, SCL/TAL-1^{-} and FLK-1^{-}, SCL/TAL-1^{+} cells. SCL/TAL-1$^{-/-}$ cells are unable to form BL-CFCs or haematopoietic cells (Faloon *et al.*, 2000; Robertson *et al.*, 2000), it is therefore possible that haemangioblasts are FLK-1^{+}, SCL/TAL-1^{+} cells (see **Figure 1.9** for a schematic of the likely expression profile of the haemangioblast).

The naturally occurring zebrafish *cloche* mutants have lowered SCL/TAL-1 levels as well as haematopoietic and endothelial defects. This indicates that a mutation in the as yet unidentified *cloche* gene has a profound impact on both these lineages, providing additional support for the possibility of their development being linked through a common ancestor (Stainier *et al.*, 1995). Genetic analysis has shown that the *scl/tal-1* locus is not linked to the *cloche* mutation (Liao *et al.*, 1998), however, the *cloche* mutation abrogates *scl/tal-1* expression. Ectopic expression of the *scl/tal-1* cDNA partially rescued the vascular and haematopoietic defects attributed to the cloche mutation indicating that *scl/tal-1* acts downstream of the cloche gene and also has a crucial role in both lineages (Liao *et al.*, 1998). Ectopic expression also results in the generation of FLK-1^{+}, SCL/TAL-1^{+} cells at the expense of other mesodermal lineages (Gering *et al.*, 1998).

1.10.4.5. VE-Cadherin

Vascular Endothelial Cadherin (VE-Cadherin)/CD144 is a calcium-dependent adhesion molecule. It is exclusively and constitutively expressed on vascular endothelium (Breier *et al.*, 1996). It is localised to the intercellular junctions where it

is crucial for vascular assembly (Vittet *et al.*, 1997). It is also known to mediate contact inhibition of endothelial cell growth and inhibit apoptosis.

As well as in mature vessels, VE-Cadherin is expressed by vascular precursors from 7.5 dpc onwards (Lampugnani *et al.*, 1992; Breier *et al.*, 1996). Early in ontogeny, VE cadherin is expressed on multipotent progenitors. However by 9.5 dpc all marked cells are destined to differentiate to form endothelial cells. *VE-cadherin*$^{+}$ cells also express *flk-1* at 9.5 dpc (Nishikawa *et al.*, 1998a). Nishikawa *et al* have used a two-dimensional culture system and cell sorting strategy to investigate the role of *VE-cadherin* in haematopoiesis. It persists into the adult vasculature and could be involved in maintaining vessel integrity. This cell marker is believed to be involved in establishing the adherence junctions of endothelial cells (Breier *et al.*, 1996). However, *VE-cadherin*$^{+}$ cells also have some lymphohaematopoietic potential (Nishikawa *et al.*, 1998b).

1.10.4.6. PECAM-1

Platelet-endothelial cell adhesion molecule 1 (PECAM-1)/CD31 was originally identified as a marker of endothelium. It is a glycoprotein from the immunoglobulin superfamily of cell adhesion molecules. It was initially found to be expressed on many cells of the vascular system besides endothelial cells including platelets, monocytes, neutrophils and some T cells (Ilan and Madri, 2003). Over time, it has been found to have key roles in numerous signalling pathways displaying a broad range of functions. It has been implicated in a diverse range of processes such as leucocyte and endothelial cell migration, maintainance of the integrity of adherens junctions, and modulation of vascular permeability (Jackson, 2003). PECAM-1 mediated cell-cell interactions are essential in angiogenesis (DeLisser *et al.*, 1997; Sheibani *et al.*, 1997; Cao *et al.*, 2002). PECAM-1 is also thought to affect the structure of the cytoskeleton, regulate catenin localisation and in turn transcription of many key players in various signalling pathways, it can itself bind several STATs and is known to directly control apoptosis and epithelium to mesenchyme transition (EMT) in the development of the cardiac cushion (Ilan and Madri, 2003).

PECAM-1 processing results in two proteins with distinct functionality. The soluble protein, comprising the receptor domain, blocks transendothelial cell migration of neutrophils, monocytes and natural killer cells *in vitro* and is known to inhibit acute inflammation *in vivo* (Liao *et al.*, 1997; Liao *et al.*, 2000). The cytoplasmic domain bears the protein's strong signalling capabilities. PECAM-1 contains intracytoplasmic immunoreceptor tyrosine inhibitory motifs (Newman *et al.*, 2001) that mediate inhibitory responses following phosphorylation via recruitment and activation of protein-tyrosine phosphatases (PTPs), typically SHP-2 (Jackson *et al.*, 1997; Hua *et al.*, 1998; Henshall *et al.*, 2001; Newman *et al.*, 2001), as well as immunoreceptor tyrosine-based activation motifs (ITAMs) (Pumphrey *et al.*, 1999). Tyrosine phosphorylation can be mediated by many mechanisms. For PECAM-1, integrin $\alpha_{2b}\beta_3$-mediated platelet aggregation, shear stress on endothelial cells, binding with the T-cell receptor antigen or integrins on extracellular matrices and stimulation with VEGF amongst others, are all known to cause this phosphorylation effect (Jackson, 2003). PECAM-1 knockout mice are viable but have detectable defects in leukocyte transmembrane membrane migration (Muller *et al.*, 1993; Vaporciyan *et al.*, 1993; Wakelin *et al.*, 1996; Duncan *et al.*, 1999).

PECAM-1 is very highly expressed in the vasculature especially the vascular endothelium and is believed to play a crucial role in vessel formation. In both rat and murine models of angiogenesis, blocking PECAM-1 expression with antibody binding prevented vessel formation in collagen I based three-dimensional cell cultures (DeLisser *et al.*, 1997). Endothelial PECAM-1 binds β-catenin (Ilan *et al.*, 1999). β-catenin phosphorylation plays a crucial role in the linkage of VE-Cadherin to the actin cytoskeleton (Ukropec *et al.*, 2002). Additionally, interactions between PECAM-1^+ cells and the extracellular matrix are know to coordinate endothelial cell proliferation and apoptosis in blood vessel formation (and subsequent regression in angiogenic remodelling) (Scatena and Giachelli, 2002).

PECAM-1 expression is also upregulated during the morula to blastocyst transition. 7 of the 8 known alternatively spliced PECAM-1 isoforms are expressed in the blastocyst although expression is restricted to cell borders. Expression is still seen in

the epiblast of the peri-implantation embryo but is undetectable by the egg cylinder stage embryo (Robson *et al.*, 2001). The cytoplasmic domain of PECAM-1 is known to have both inhibitory and activating functions in cell signalling (Ilan *et al.*, 1999; Newton-Nash and Newman, 1999). PECAM-1 could therefore have a role in maintaining pluripotency. PECAM-1 is known to bind SHP-2. SHP-2 binding to gp130 enhances ES cell self-renewal (Burdon *et al.*, 1999) therefore PECAM-1 could modulate SHP-2 availability to gp130.

VEGF is known to induce the phosphorylation of adherens junctions including β-catenin (Ilan *et al.*, 1999; Abedi and Zachary, 1997; Esser *et al.*, 1998). Phosphorylation of β-catenin results in an increase in its association with PECAM-1. This complex is believed to have a variety of roles including the modulation of endothelial cell integrity and permeability (Graesser *et al.*, 2002). Tyrosine phosphorylation of β-catenin, along with other adherens junction components is known to result in a loss of cellular adhesion in the vascular endothelium (Daniel and Reynolds, 1997; Provost and Rimm, 1999). This could influence the migration of vascular endothelial precursors in early development and in angiogenesis.

PECAM-1 is lost during epithelial to-mesenchymal transformation (EMT) in endocardial cells of the heart resulting in the formation endocardial cushion. As these PECAM-1 expressing cells lose their expression, they begin to acquire αSMA expression. This process is thought to be stringently regulated by VEGF, with slight variation in VEGF levels affecting the normal course of cardiac cushion EMT and in turn, cardiac development (Enciso *et al.*, 2003; Miquerol *et al.*, 2000)

1.10.4.7. Other Potential Haemangioblastic Markers

1.10.4.7.1. Runx1

The coordinated emergence of endothelial and haematopoietic precursors in YS blood islands is not seen intraembryonically where the dorsal aorta forms before the emergence of HSC. This implies a different haemangioblast or route of progenitor differentiation. The AGM has been observed to display overlapping expression of

endothelial and haematopoietic markers on cells that form from the ventral walls of the dorsal aorta (Marshall and Thrasher, 2001).

Runx1 (*cbfa2*, AML-1, PEBP2) is a heterodimeric transcription factor, which contains a *runt*-homology domain (Speck *et al.*, 1999; Tracey and Speck, 2000). It is expressed in the YS mesenchyme at 7.5 dpc. It is expressed in erythrocytes and the endothelium at 8 dpc but its expression drops after this time and is expressed in the AGM at 8.5 dpc in ventral aortic endothelial cells (North *et al.*, 1999). The mesenchyme and endothelium in the larger blood vessels express r*unx1* thereafter as well as haematopoietic clusters arising in the arteries. *Runx1* has been shown to be required in the development of HSC in AGM but not in other regions e.g. vasculature in the YS where haematopoiesis first emerges (Wang *et al.*, 1996; Okuda *et al.*, 1996; Cai *et al.*, 2000; Mukouyama *et al.*, 2000). At 10 dpc, *runx1* is expressed in haematopoietic cell clusters and endothelial cells of the vitelline and umbilical arteries and the ventral wall or the dorsal aorta (North *et al.*, 1999).

Runx1 knockout mice have normal YS haematopoiesis but no foetal liver haematopoiesis (Okuda *et al.*, 1996; Wang *et al.*, 1996; North *et al.*, 1999;). North *et al.* (1999) showed that all HSCs were $RUNX1^{+}$ cells, expressing endothelial markers, which supports the concept of the haemogenic endothelium theory. *Runx1* therefore has a role in the establishment of definitive haematopoiesis and if YS progenitors do migrate to intraembryonic sites for maturation and further development, *runx1* expression may be implicated in setting up a homing signal to the AGM or foetal liver sites of development. Haploinsufficient *runx1* mutants have the unusual phenotype of elevated numbers of HSC at 10 dpc in the YS and AGM. These hint that *runx1* is involved in cell expansion and differentiation.

1.10.4.7.2. Podocalyxin-Like Protein 1

Podocalyxin-like protein 1 (PCLP1) is another candidate marker of the haemangioblast. Structurally, it is similar to CD34 and is expressed on endothelial and haematopoietic cells (Hara *et al.*, 1999). These authors showed that $PCLP1^{+}$, $CD45^{-}$ cells could form both lineages when cultured on stromal cells and also

contributed to cells from both lineages in neonatal myeloablated mice. This cell population appears endothelial, expressing FLK-1, CD34, CD31 with the ability to take up acetylated low-density lipoproteins (acLDL), however, in keeping with the hypothesis regarding the haemogenic potential of endothelium (Nishikawa *et al.*, 1998a; Nishikawa *et al.*, 1998b; Ogawa *et al.*, 1999), these endothelial-like cells also have an observed capacity to differentiate to form haematopoietic cells in conditioned neonatal mice. This population was heterogenous however and $PCLP1^{+}$ cells will need to be analysed clonally for their potentiality and accompanied expression profile to ascertain if PCLP1 is actually marking haemangioblastic cells.

1.10.5. The Haemangioblast Hypothesis

This constant change in haematopoietic development in the embryo emphasises the dynamism of the system from its inception. Though the gradual shifts in haematopoiesis are beginning to be understood, the complicated signalling that takes place in order to trigger constant change with control and direction of progenitor cells has eluded complete understanding thus far. The close developmental association between haematopoietic and endothelial cells has led to a well-established hypothesis suggesting that the lineages originate from a common precursor.

Existence of a common precursor to haematopoietic and endothelial cells was first postulated by Sabin (1920) from precise observations of haematopoietic development of chick blastoderms (**Figure 1.5**). This common precursor was given the name of haemangioblast by Murray (1932) following observations of YS haematopoiesis and blood islands generation, again in the avian model. Though unproven, the existence of the haemangioblast has been embraced and the theory unchallenged for nearly a century.

Dieterlen-Lièvre and colleagues have observed that in some chick-quail chimaeras, groups of intramesodermal haematopoietic cells were of one or the other species (Martin *et al.*, 1980) suggesting that each group arose through multiplication of one original cell. More recently Hirschi and Goodell (2001) have suggested the

maintenance of the haemangioblastic population through to adulthood in the "side population" cell (Goodell *et al.*, 1996) that has been observed to contribute to both haematopoietic and vascular repair.

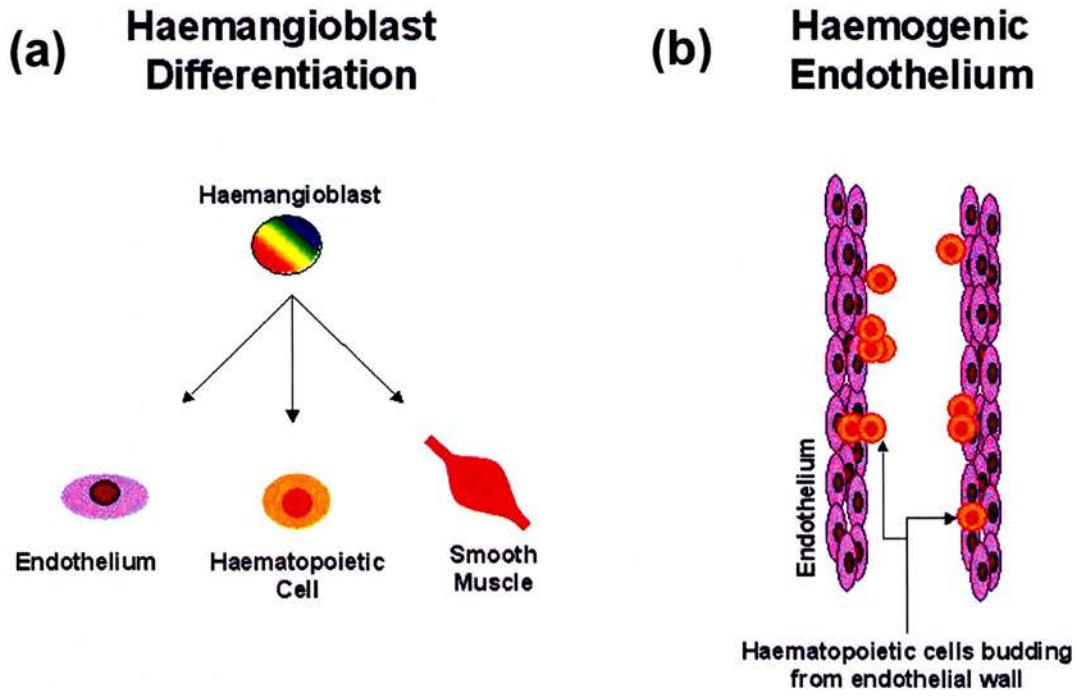

Figure 1.8 *Schematic diagram depicting the two hypotheses suggested for the differentiation of the vascular lineages. In* ***(a)*** *the haemangioblast is the precursor to all three vascular lineages whereas in* ***(b)*** *the endothelial lining of the blood vessels have the capability to differentiation to haematopoietic progenitor cells which bud off the vascular wall.*

1.10.6. The Haemogenic Endothelium

In 1916, Jordan was able to localise this haemogenic activity to the ventral surface of major blood vessels and hypothesised that vessel branching typically took place from the ventral surface, indicating that a more primitive stem cell population was maintained along the ventral wall of blood vessels. Histological analysis of vascular endothelium also showed that cells were budding from the endothelial wall (Smith and Glomski, 1982; Tavian *et al.*, 1996). More recent research in several model species has clarified that it is haematopoietic cells that bud off the endothelial walls of the ventral wall of the dorsal aorta (**Figure 1.8**). This indirectly supports the theory for the existence of the haemogenic endothelium (Pardanaud *et al.*, 1996; Jaffredo *et al.*, 1998; North *et al.*, 1999; Tavian *et al.*, 1996; Tavian, 1999). Critical to the viability of the haemogenic endothelium hypothesis will be clarification of

whether the haematopoietic cells are budding off endothelium, or if circulating haematopoietic cells are adhering to endothelial surface of blood vessels.

It is a possibility that in definitive haematopoiesis, haematopoietic cells arise from the cells in the endothelium of the AGM: "haemogenic endothelial cells", but that in primitive haematopoiesis, they are derived from the putative haemangioblast. Beyond the *in vitro* evidence and the histological data *in vivo* that suggest its possible existence, haemangioblasts or haemogenic endothelia have never been isolated or identified *in vivo*.

1.10.6.1. Experimental Evidence in Support of the Existence of the Haemangioblast

To check whether haematopoietic cells were actually budding off endothelial cells Jaffredo *et al.*, (1998) injected fluorescent acetylated low-density lipoproteins (acLDL) into embryonic chick hearts before aortic haematopoietic clusters could be observed so that the endothelium was tagged. They found that the clusters of haematopoietic cells that appeared thereafter were also fluorescent and thus likely to have divided from the endothelial cells.

Ogawa *et al.* (2001) have an interesting hypothesis to explain the possibility of there being different developmental pathways for the generation of haematopoietic progenitors. They suggest that 2 distinct pathways for differentiation exist and that definitive haematopoiesis only utilises one but YS haematopoiesis uses both. Their model accommodates the possible existence of the haemangioblast and the haemogenic endothelium. The primitive haematopoietic system, arising in the YS of a developing embryo is generated from the haemangioblast or a distinct population of mesodermal cells. The definitive lineage – be it derived from the YS or arising in the AGM, is derived from endothelial cells with a haemogenic capacity. They suggest that not only do haematopoietic cells arise from further differentiating endothelial precursors, but that endothelial cells provide the environment for the maturation of the haematopoietic precursors (Ogawa *et al.*, 2001).

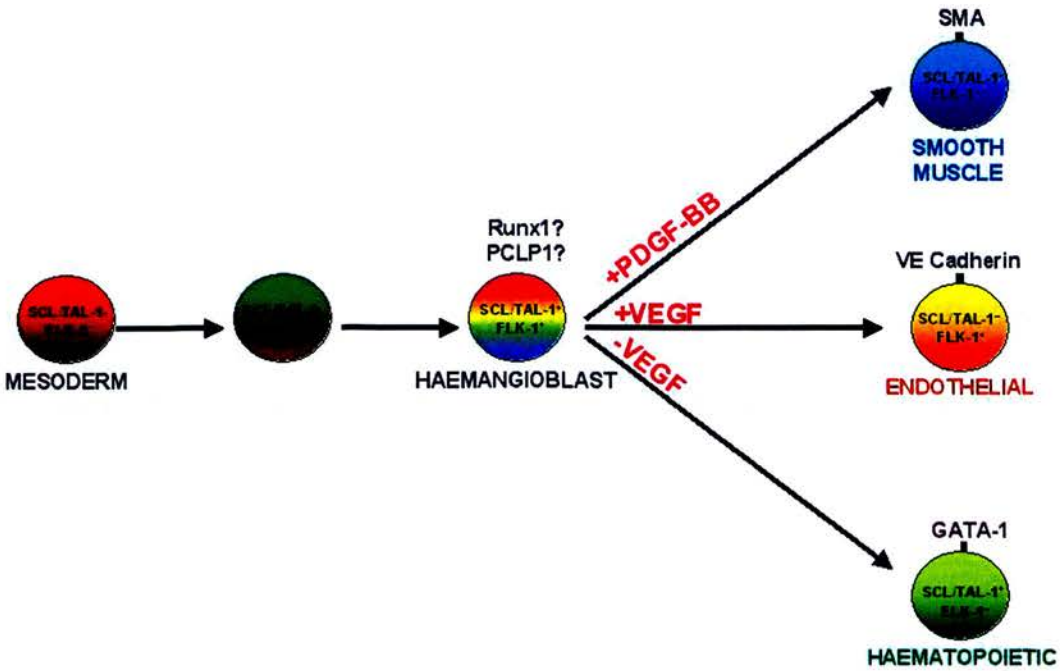

Figure 1.9 *Proposed course of differentiation of the haemangioblast to the lineages that comprise the haematopoietic system based on current discoveries. The expression patterns for FLK-1 and SCL/TAL-1 are stated for each cell stage. Definitive markers of the vascular lineages are included, as are potential haemangioblast markers.*

1.10.7. The Haemangioblast and Mural Cells

Endothelial cells line the vessel lumen and mural cells (Sone *et al.*, 2003) associate with the outer endothelium (D'Amore, 2000). The interaction of the two cell types is key in vascular development and function. This relationship has recently been exemplified in culture. FLK-1$^+$ cells derived from differentiated ES cells have been found to express a marker for mural cells in culture: αSMA. When in the presence of VEGF, *flk-1*$^+$ cells tested positive for platelet endothelial cell adhesion molecule 1 (PECAM-1), an endothelial cell marker; these cells were surrounded by αSMA$^+$ cells. *Flk-1*$^+$ cells obtained from embryos behaved in the same way. αSMA positive cells were seen to attach to PECAM-1$^+$ endothelial tubes. A lumen then formed with the cells within the lumen testing positive for blood cell markers (CD45 and Ter119). It was believed that in the absence of VEGF signalling, FLK-1$^+$ cells would form mural cells via PDGF-BB signalling as a default. PDGF-BB is required for vascular smooth muscle and pericyte proliferation and migration (Hellstrom *et al.*, 1999). Mice deficient in PDGF-BB die in the latter stages of ontogeny from haemorrhaging

and capillary rupture due to the lack of vessel-associated cells (Lindahl *et al.*, 1997). This indicates an alternative pathway for mural cell differentiation and a different role for PDGF-BB. This is backed up by data from PDGF-BB$^{-/-}$ mice that still had αSMA expression on their mural cells. This was established *in vitro* whereby FLK-1^{+} cells were exposed to either VEGF or PDGF-BB. When exposed to both simultaneously, development of both endothelial and mural cells was enhanced. This shows that cell fate is determined to a large extent by the exposure to growth factors at timed intervals (Yamashita *et al.*, 2000). When VEGF was applied to cells, *flk-1* was maintained. If there was no VEGF, mural cells developed, and *flk-1* expression was lost gradually. In a positive feedback loop, it seems as though VEGF is required for the maintenance of *flk-1* expression and hence differentiation down the endothelial lineage. Without VEGF, *flk-1* expression is lost and the mural cell fate becomes the default (Yamashita *et al.*, 2000). These discoveries may suggest that endothelial cells have the ability to transdifferentiate to mural cells in response to PDGF-BB or an as yet unknown signal. It is also possible that a separate population of mural cells share a common precursor with endothelial cells. This may well be a sign that the haemangioblast is multipotent. **Figure 1.9** shows a schematic of haemangioblast differentiation and the likely expression profile of haemangioblastic cells and their derivatives.

1.11. Summary

- Cell-based therapies are increasingly being investigated as the knowledge of the behaviour of and differentiation procedures for ES cells increases. The possibility of carrying out cellular replacement has been strengthened following the successful isolation of ES cells from humans.

- This project was designed to address the possibility of differentiating an early, multipotent progenitor population from mES cells, purifying this population and assessing whether such a population can engraft *in vivo* and perform the range of functions expected from its endogenous counterpart.

- The progenitor being differentiated was the haemangioblast: the putative progenitor of all the vascular lineages. This progenitor has never been isolated or identified in development, but both *in vivo* and *in vitro* data have supported the case for its existence.

- The FLK-1 receptor tyrosine kinase is the earliest known marker to segregate cells designated to form vascular tissues from multipotent mesoderm. The *flk-1* gene was chosen for development of the targeting strategy (Chapter 3), that enables the identification and isolation of a pure FLK-1^+ cell population, following directed differentiation for haemangioblastic cell fates. Chapters 4, 5 and 6 summarise experiments carried out with targeted cells to develop the best possible differentiation regime to isolate haemangioblastic cells and the *in vivo* transplantation of the purified cells to assess their viability, contribution and function.

CHAPTER 2

Materials and Methods

Material and Methods

2.1. Molecular Biology Methods

2.1.1. Culture Media

2.1.1.1. Bacterial Growth Medium

For the routine growth of bacteria for the propagation of plasmid DNA Luria Bertani (LB) medium was made up as follows: 10% (w/v) tryptone, 5% (w/v) yeast and 5% (w/v) sodium chloride (NaCl) were dissolved in deionised water and autoclaved in an airtight container. If selection for bacterial cells containing plasmid DNA was required, the medium was supplemented with an antibiotic for which the plasmid being propagated conferred a resistance – commonly ampicillin (50μg/ml) or kanamycin (33μg/ml)

2.1.1.2. LB Agar Plates

For selective growth of bacterial cells containing plasmid DNA agar plates were used. Plates were supplemented with an antibiotic for selection of bacterial cells containing a plasmid with a resistance gene for the antibiotic being supplemented. (LB medium components supplemented with 0.15g/ml agar, mixed and made up to one litre in an airtight container and autoclaved) with the appropriate antibiotic selection added to the agar – commonly either ampicillin (100μg/ml) or kanamycin (33μg/ml). Agar plates were supplemented with filtered antibiotic solutions either after autoclaving or once solidified agar had been melted in a microwave oven. The agar was cooled until the container was hot to touch but so much that it could be handled - approximately 50-55°C. At this point, the appropriate concentration of antibiotic was added to the agar and mixed. The agar was poured onto sterile plates in a laminar flow hood or by a Bunsen burner flame, covered, and stored at 4°C for up to a month.

2.1.2. Plasmid DNA propagation

2.1.2.1. Transformation

In order to propagate plasmid DNA, the circular DNA was transformed into a line of *Eschericia coli* suitable for simple transformations or efficient subcloning steps: Subcloning Efficiency DH5α™ Chemically Competent *E.coli* (Invitrogen™). [Genotype: F^-ϕ80*lac*ZΔ ΔM15(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-].

100µl of competent cells were thawed on ice, placed in a 1.5ml microcentrifuge tube and gently mixed with a pipette tip. 10-20ng of DNA (in a maximum volume of 5µl) were added to the cells and mixed in. This mixture was incubated on ice for 30 minutes. The cells were then heatshocked for 45 seconds in a 37°C water bath. This action temporarily permeabilised the cell membrane of the cells, thus allowing the passage of the plasmid DNA into the cells. The cells were placed on ice for a minimum of 2 minutes to recover. 900µl of pre-warmed (37°C) LB medium was added to the microcentrifuge tube, which was then incubated for 1 hour in a 37°C water bath. 25 and 50µl of the transformation culture were plated on LB agar plates. The plates were incubated upside down (to prevent drying of the plates) at 37°C overnight. The following day, the plates were checked for colonies of bacteria. Surviving colonies had antibiotic resistance conferred upon them by the selection marker in the transformed plasmid. Single colonies could then be picked and grown in LB medium for plasmid propagation.

2.1.2.2. Isolation of High Concentrations of Plasmid DNA

The QIAGEN® Maxi kit for plasmid purification was used. This kit is designed for the isolation of up to 100µg of plasmid DNA using an anion-exchange resin that both binds and allows the elution of DNA depending on the pH and salt concentrations of the buffers passed through the resin at the time. The advised procedure outlined in the handbook was adhered to.

A single colony was picked from an agar plate of bacterial cells transformed with the desired plasmid using sterile micropipetter tips and placed in 15ml tubes containing

3ml of LB medium and the appropriate antibiotic selection. The tubes were incubated at 37°C with moderate (200-250 revolutions per minute (rpm)) shaking for approximately 8 hours. This starter culture was then poured into 400ml of sterile LB medium with the same selection. The flask used for growth had a volume of at least 4 times the volume of the culture medium that it contained. This ensured that the bacterial culture was provided with sufficient oxygen for growth. The culture was incubated for 12-16 hours (overnight) at 37°C with shaking.

The bacterial cells were harvested by centrifugation at 6000*g* [≅6000rpm] for 15minutes at 4°C (Du Pont Instruments Sorvall® RC-5B refrigerated superspeed centrifuge, F-16/250 rotor). The supernatant was poured away and the pellet resuspended in 10 ml of Buffer P1 (50 mM Tris(hydroxymethyl)aminomethane-hydrochloride (Tris-HCl), pH 8.0; 10mM ethylenediaminetetraacetic acid, disodium (EDTA), 100µg/ml RNase A). 10ml of Buffer P2 (200mM sodium hydroxide (NaOH); 1% sodium dodecyl sulphate (SDS) (w/v)) was added for cell lysis and the container inverted 4-6 times to mix the solutions. The vessel was left at room temperature for 5 minutes. 10ml of chilled (4°C) Buffer P3 (3M potassium acetate pH 5.5) was added to the container and mixed through immediately by inverting the tube 4-6 times to neutralise the lysis buffer. It was incubated on ice for 20 minutes before centrifugation at 20000*g* [12000 rpm] for 30 minutes at 4°C. The supernatant was collected after centrifugation and passed through Whatman 3MM filter paper to remove all traces of precipitated proteins.

A QIAGEN-tip 500 was equilibrated by the addition of 10 ml of Buffer QBT (750mM NaCl; 50mM 3-(N-morpholino)-propanesulphonic acid (MOPS), pH 7; 15% isopropanol (v/v)) to the column and letting it pass by gravity flow. Once the equilibration buffer had emptied from the column, the filtered supernatant was added to the column and also allowed to pass through completely. The column was washed with two 30ml applications of Buffer QC (1M NaCl; 50mM MOPS, pH 7.0; 15% isopropanol (v/v)). The DNA was eluted in 15ml of Buffer QF (1.25M NaCl; 50mM Tris-HCl, pH 8.5; 15% isopropanol (v/v)) in a glass Corex tube. To this, 10.5 ml of isopropanol was added and mixed by inversion of the tube in order to precipitate the

DNA. The tube was centrifuged immediately at 15000*g* [10000 rpm] for 30 minutes at 4°C (Du Pont Instruments Sorvall® RC-5B refrigerated superspeed centrifuge, HB-6 rotor). The supernatant was carefully decanted off and the pellet washed with 70% ethanol (v/v) and re-centrifuged at 15000*g* [10000 rpm] for 10 minutes at 4°C. The ethanol was pipetted off carefully and the pellet left to air-dry at room temperature until the edges of the pellet looked transparent under light. The dried pellet was resuspended in an appropriate volume of TE buffer (10mM Tris-HCl, pH 7.5; 1mM EDTA, pH 8), usually 500µl and quantified using UV spectrophotometry.

2.1.2.3. Isolation of Low Concentrations of Plasmid DNA

When small-scale propagation of plasmid DNA was required e.g. to obtain enough DNA to carry out digests to check multiple colonies for successful ligation events from a subcloning step, the Wizard® Plus Miniprep DNA Purification System (Promega) was used. This kit relied on the binding capability of DNA to a silica membrane to purify plasmid DNA.

As with the maxiprep cultures, single colonies of bacteria were picked off agar plates. Each colony was put into 3mls of LB medium in a 15ml screw-capped tube. The cultures were incubated overnight at 37°C with moderate shaking. The following day the cultures were spun down in a centrifuge (Eppendorf centrifuge: 5810R) at 3220g [4000rpm] for 10 minutes. The supernatant was poured off and the pellet resuspended in 300µl of Cell Resuspension Solution (50mM Tris-HCl, pH 7.5; 10mM EDTA; 100µg/ml RNase A). The resuspended pellet was transferred to a 1.5ml microcentrifuge tube. 300µl of Cell Lysis Solution (0.2M NaOH; 1% SDS (w/v)) was added and mixed by inverting the tube several times. 300µl of Neutralization Solution (1.32M potassium acetate, pH 4.8) was added immediately after and mixed by inverting the tube, as before. The lysate was centrifuged at 13000*g* [13000rpm] in a microcentrifuge[1] for 5 minutes. Meanwhile, a vacuum manifold (Promega Vac-Man®) was set up. The minicolumns designed for use with the vacuum manifold were attached to the apparatus together with the syringe barrel

[1] Various different microcentrifuges were used. Each had a radius (r) of approximately 70mm. Conversions from revolutions per minute (rpm) to relative centrifugal force (rcf) were carried out using the following equation: $\mathbf{rcf=1.11 \times 10^{-6}(rpm)^2 r}$ (Sambrook & Russell, 2001)

attachment for the minicolumns. One column was required for each miniprep. 1ml of resuspended resin (40% (v/v) isopropanol, 4.2M guanidine HCl) was pipetted into each minicolumn barrel. The cleared lysates from the bacterial cultures were loaded into the barrels and allowed to flow through the column with the resin using the vacuum. 2ml of Column Wash Solution (80mM potassium acetate; 8.3mM Tris-HCl, pH 7.5; 40μM EDTA; 55% (v/v) ethanol) were then applied to the barrels and allowed to pass through. The vacuum was maintained on the columns for 30 seconds to dry the resin in the columns. The barrels were removed from the columns and discarded. The minicolumns were placed in 1.5ml microcentrifuge tubes and spun in a microcentrifuge at 13000*g* [13000rpm] for two minutes to ensure that there was no liquid remaining in the columns. The minicolumns were then transferred to new microcentrifuge tubes. 50μl of sterile ddH_2O (distilled and deionised water) were added to each column and left for one minute to enter the resin. The microcentrifuge tubes and minicolumns were then spun for one minute at 13000*g* [13000rpm] to elute the DNA. Using 3ml overnight cultures, approximately 10μg of plasmid DNA was a typical yield.

2.1.3. DNA Quantification

2.1.3.1. UV Spectrophotometry

UV Spectrophotometry works on the principle that both purines and pyrimidines are able to absorb ultraviolet light. The Beer-Lambert law states that for a parallel beam of monochromatic radiation passing through a homogeneous solution the absorbance is proportional to the product of the concentration and pathlength. Nucleic acids absorb UV light maximally at 260nm wavelength so the optical density (OD) i.e. the drop in light detected as a result of absorption by the nucleic acid molecules was measured at this wavelength. A UNICAM 5625 UV/VIS spectrophotometer was used. At the 260nm wavelength, an OD measurement of 1 corresponds to 50μg/ml for double-stranded DNA and 40μg/ml for single-stranded DNA or RNA. To derive the actual concentration of a DNA solution from a spectrophotometer reading, the following calculation was carried out:

OD_{260} x Reading Adjustment* x Dilution factor = Number µg/ml

*(dsDNA=50; ssDNA/RNA=40)

By determining the ratio from absorbance readings at 260nm and 280nm, a measure of DNA purity can be determined. DNA and proteins absorb light differently at these wavelengths therefore their relative measurements give an estimate of the amount of protein contaminating a DNA sample. Conventionally an OD_{260}/OD_{280} of greater than 1.6 is desirable. UV spectroscopy was suitable for quantification of DNA at high concentrations: typically at least 1µg/ml. This requirement for accuracy limited its use to the quantification of maxipreps of plasmid DNA and the quantification of genomic DNA.

For routine quantification of small amounts of plasmid DNA, between 1-5µl of plasmid DNA was run on an ethidium bromide agarose gel alongside a size marker, typically the BIOLINE Hyperladder 1 1 kilobase pair (kb) ladder was used. The band intensity of the DNA being quantified was taken to have the same concentration as the band on the standardised size marker, which had the most similar level of fluorescence under UV light, indicating a similar incorporation of ethidium bromide within the DNA structure and thus a similar concentration of DNA.

2.1.4. Electrophoresis

2.1.4.1. Horizontal Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to visualise DNA being manipulated and to isolate particular DNA fragments for cloning steps or electroporation into cells, as well as a way of quantifying DNA concentrations. Powdered agarose was mixed with 1x TAE (Tris-acetate-EDTA) electrophoresis buffer (40mM Tris base (tris(hydroxymethyl)methylamine), 1mM EDTA, pH 7.7 (with glacial acetic acid)) and heated to melt the agarose in the TAE buffer. By altering the concentration of agarose used in the gel, the density of the agarose gel matrix was modified for better separation of large (low percentage) or small (high percentage) fragments. For

routine gels, 1% agarose (w/v) was used. Once the agarose was fully melted and allowed to cool until the agarose seemed more viscous, ethidium bromide (0.25μg/ml) was added to the solution before it was poured into a gel-casting tray and left to set with combs inserted into the agarose to form the wells. For the resolution of DNA fragments that were smaller than 1 kb in size, 2%-4% (w/v) agarose gels were made and run in TBE (Tris-borate-EDTA) buffer (89mM Tris, 2mM EDTA, pH to 8.3 with orthoboric acid). TBE has a higher buffering capacity but a lower conductivity than TAE. Whereas TAE is better at resolving fragments larger than 2.5kb where size separation is faster, TBE is more suited to the resolution of smaller fragments as TBE gels can be run successfully at higher voltages. The gels were immersed in 1x TAE or TBE buffer before the DNA samples and appropriate size marker were loaded and the gel run in electrophoresis apparatus. Both the DNA samples and markers (if not prepared with loading buffer) were first mixed with gel-loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 15% (w/v) Ficoll, dissolved in water) to increase the density of the samples and ensure that DNA was weighted down into the wells and did not disperse into the running buffer. Gels were typically run at 80-100 volts (V) until good band separation was achieved.

2.1.4.2. Pulsed Field Gel Electrophoresis (PFGE)

DNA separation was problematic on conventional agarose gel electrophoresis apparatus with a linear charge field for DNA fragments of 15-20 kb and greater which are hard to resolve due to their mobility through an agarose matrix being similar. Fragments of this size therefore tended to migrate together. In PFGE, the DNA is forced to change direction continuously during electrophoresis due to a constant shift in the direction of the current in the gel. Each time the electric field is changed in direction, slightly smaller DNA fragments are more likely to move towards the new anode than larger ones. Carried out frequently over a long period of electrophoresis, separation of large DNA fragments from each other is enhanced and individual bands become more discrete. The contour-clamped homogeneous electric field (CHEF) (Chu *et al.*, 1986; Chu *et al.*, 1990) apparatus (CHEF-DR® III System, BIORAD) was used. 1.2% (w/v) TAE gels were used in 0.5x TAE buffer. Ethidium

bromide staining was carried out after the run as incorporation of ethidium bromide into large fragments of DNA could have distorted the migration of the fragments during electrophoresis. Pulsed field gels to resolve fragments of 17 and 18 kb in size were run at 9V, 120° reorientation gradient, 0.1 second pulse time for 6 hours at a constant temperature of 14°C.

2.1.5. DNA Purification Methods

2.1.5.1. Purification of DNA from Agarose Gels

A conventional agarose gel of a restriction enzyme digest isolating the required piece of DNA was run until the desired DNA fragment was clearly separated from all other fragments. Under long wave UV light (365nm) which is less damaging to the DNA than short wave UV light (302nm) which is conventionally used to visualise ethidium bromide stained DNA, the agarose was cut to isolate the band. DNA fragments isolated from gels were usually needed for subcloning steps and the integrity of the fragments' ends needed to be maintained if the overhangs were to be used in a ligation step, so the procedure was carried out as quickly as possible to ensure minimal damage to the DNA. The agarose containing the DNA was weighed and divided into 1.5ml microcentrifuge tubes – each with a maximum agarose weight of 380mg.

If the size of the DNA fragment to be purified was below 10 kb the QIAquick® Gel Extraction Kit (QIAGEN®) was used for gel purification. This kit made use of a spin column lined with a silica-gel membrane that binds DNA under high salt concentrations and low pH allowing DNA contaminants to pass through the column. Elution is favoured under low salt concentrations and high pH. Three volumes of buffer QG (components not given) were added to the gel fragment (1g weight of agarose was taken to represent 1ml). The microcentrifuge tube containing the gel fragment was incubated at 50°C for 10 minutes or until the agarose had completely dissolved. Throughout this incubation, the tube was inverted regularly to help the gel dissolve. After the agarose had dissolved, one gel volume of isopropanol was added to the sample. This was to aid the yield of DNA fragments either smaller than 500 bp or larger than 4 kb as it enhanced the binding properties of the column. A

QIAquick spin column was placed in a 2ml collection tube provided with the kit. The dissolved agarose and DNA mixture were applied to the column, which was then centrifuged for 1 minute at 13000*g* [13000 rpm] in a microcentrifuge. This bound the DNA to the spin column whilst allowing the dissolved agarose to pass through the column. The flow-through was discarded and 500µl of buffer QG was added to the spin column and the column spun again. This step ensured that all residual agarose was removed from the spin column and hence the DNA. The column was washed with 750µl of buffer PE (components not given) and recentrifuged for 1 minute. The flow-through was discarded and the spin column replaced in the collection tube and re-spun to ensure that the resin in the spin column was dry and free from any ethanol remaining from the wash buffer. After this spin, the column was placed in a new 1.5ml microcentrifuge tube. To elute the DNA, 30µl of buffer EB (10mM Tris-HCl, pH 8.5) was added to the column and left to soak into the resin for 1 minute. The column was then spun at 13000*g* [13000rpm] for one minute and the DNA collected in the microcentrifuge tube.

If the fragment was greater than 10 kb in length, the use of this kit was not as efficient, possibly due to the risk of shearing larger DNA fragments during the procedure so the Schleicher and Schuell Elu-Quik DNA Purification Kit which was suitable for the purification of fragments up to 200 kb in length was used. It works by exploiting the ability that DNA has to bind glass in sodium perchlorate. After isolation of the DNA fragment in agarose, binding buffer (sodium perchlorate – molarity not given) was added at 2.8x the volume of agarose to the agarose in the microcentrifuge tubes. The tube was incubated in a 50°C water bath for 5 minutes, or until the agarose had completely melted in the binding buffer. The tube was periodically inverted during this time. The glass concentrate stock (glass beads in sodium perchlorate – molarity not given) was resuspended by vigorous pipetting before 20µl/100mg of agarose was added to the melted agarose suspension. The tube was placed on a rotator (Stuart Scientific, Rotator SB1) at room temperature for 10 minutes to ensure the glass beads circulated throughout the tube. The tube was centrifuged at 5000*g* [8000rpm] for 1 minute in a microcentrifuge. The supernatant was discarded and 500µl of wash buffer (ethanol based – exact components not

given) was added to the tube. Using pipetting, the glass bead pellet was resuspended in the wash buffer and the tube inverted several times. The tube was centrifuged as before and the supernatant discarded again. Another 500µl wash was carried out and the tube was centrifuged for 1 minute once more. 500µl of salt reduction buffer (ethanol based – exact components not given) was added and the pellet resuspended in the buffer. The tube was inverted several times and centrifuged at 5000*g* [8000rpm] in a microcentrifuge for 1 minute. The supernatant was discarded and the pellet centrifuged again to draw out as much liquid from the pellet as was possible. Any extra supernatant that was drawn from the pellet was removed with a micropipettor. The tube was left open for 20-30 minutes at room temperature to dry the pellet. 10µl of sterile ddH_2O per 20µl of glass bead concentrate added previously was added to the glass bead pellet. The pellet was resuspended in the water by flicking the tube several times. The mixture was incubated at 50°C for 5 minutes with occasional flicking of the tube to release the DNA from the glass beads into the water. The tube was centrifuged at 5000*g* [8000rpm] for one minute to separate the DNA in the supernatant from the glass bead pellet. The supernatant was removed to a new 1.5ml microcentrifuge tube and spun again to pellet any residual glass concentrate that would have been carried over when the supernatant was removed from the glass pellet. The supernatant was removed from any pellet that may have formed and placed in a new microcentrifuge tube. The DNA was stored at –20°C.

2.1.5.2. Phenol:Chloroform Purification of DNA

To remove any proteinaceous matter from a sample of DNA, a phenol:chloroform extraction was carried out. Phenol binds to any protein in the sample, the chloroform, when mixed with phenol, forms an organic mixture of high enough density to be drawn down when centrifuged forming 2 distinct phases, a lower organic one of high density, the upper one being a purified DNA solution. The protein – having been drawn away from the DNA sample by the phenol forms a visible interphase between the organic and pure DNA phase. Typically DNA samples were made up to 200µl with TE buffer in a 1.5ml microfuge tube. 100µl of phenol and 100µl of chloroform were both added to the DNA sample. The mixture was vortexed for 30 seconds to thoroughly mix the solvents and the DNA sample.

The mixture was spun at 13000*g* [13000rpm] in a microcentrifuge for 10-15 minutes. The top aqueous phase was removed from the tube. Great care was taken not to interrupt the interphase containing the protein contaminant or the lower organic phase. When very high purity DNA was required, a second step was carried out. An equal volume of chloroform was added to the isolated aqueous phase from the previous step. The mixture was vortexed as before and centrifuged. Again the top aqueous phase was retained. As the volume of aqueous phase containing the DNA was usually large, the phenol:chloroform step was usually followed by an ethanol precipitation step to concentrate the DNA sample.

2.1.5.3. Ethanol Precipitation of DNA

To the DNA sample being precipitated, 0.1x the volume of 3M sodium acetate, pH 5.2 was added and mixed in a 1.5ml microcentrifuge tube. To this mixture, 2 volumes of -20°C 100% ethanol was added and mixed with the tube. The sample was incubated at -20°C for 1-2 hours to ensure maximal precipitation of DNA. The sample was then spun at 13000*g* [13000rpm] for 10 minutes to pellet the precipitated DNA. The supernatant was removed and the pellet washed in room temperature 70% ethanol (v/v). The sample was spun again at 13000*g* [13000rpm] for 5 minutes. The ethanol was removed from the pellet, which was left to air dry in the microcentrifuge tube until the edges of the pellet had turned transparent under light. The DNA pellet was resuspended in an appropriate volume of sterile distilled, seionised water (ddH_2O) or TE buffer.

2.1.6. DNA Manipulation and Modification

2.1.6.1. Restriction Endonuclease Digestion

Restriction endonuclease digests were used for the excision of fragments for subcloning steps, for verification of successful ligations, propagation steps or in Southern blotting to identify targeting events. The manufacturer's instructions were followed (Roche in most instances) however, despite the fact that 1 unit (U) of standard enzyme is shown to cleave one μg of DNA at optimal temperature for cleavage (37°C for the majority of enzymes) in 1 hour, often a 5-10 fold excess was used in the reactions (enzymes came in a minimum10U/μl concentration so it was more convenient for individual digests to pipette an excess) and incubation times were greater to ensure good digestion. Restriction enzymes are preserved in a 50% glycerol solution. An enzyme volume maximum of 10% of the total reaction volume was never exceeded so as to avoid a glycerol concentration of greater than 5%, which may have inhibited the digestion of DNA. Typical reaction examples for both plasmid and genomic DNA are given below.

GENOMIC DNA

(e.g. for Southern analysis)

10μl Genomic DNA (1μg/μl)

3 μl 10x reaction buffer (Supplied by enzyme manufacturer)

2μl High Concentration (40U/μl) Restriction Enzyme

15μl ddH_2O

TOTAL=30μl

Incubation was carried out in a water bath set at the optimal temperature for enzyme activity. The reaction was spiked the next day with 1μl of 40U/μl enzyme and left for 3-4 hours.

NB. A small reaction volume needed to be maintained in order for the entire sample to be loaded into one well on an agarose gel.

PLASMID DNA

(e.g. for subcloning steps)

10µl Plasmid DNA (1µg/µl)

5µl 10x reaction buffer (Supplied by enzyme manufacturer)

1µl Restriction enzyme (10U/µl)

34µl ddH_2O

TOTAL=50µl

Incubation was typically for 1-2 hours at the optimal temperature for enzyme activity.

Often it was required that a plasmid be digested with two different enzymes. If the two enzymes did not share the same reaction buffer for optimal digestion, it was sometimes possible to find a buffer that would cut both to at least 75% efficiency, which could be used. In such cases, the compromised enzyme was added at a higher concentration. However, sometimes this was not the case and the two enzymes could not be used together in digests. In these instances, sequential digests needed to be carried out whereby one reaction was carried out and the sample purified by phenol:chloroform purification and ethanol precipitation before the next enzyme digestion could be carried out. Most restriction enzymes could be heat inactivated with a 15-minute incubation at either 65°C or 75°C. The few that could not be inactivated, required a phenol:chloroform extraction followed by an ethanol precipitation for the removal of the enzyme to stop the activity.

2.1.6.2. Partial Digestion

When ablation of one of several identical restriction endonuclease sites is required, a partial restriction digest needs to be carried out to isolate a DNA fragment where the required restriction site is the only one cleaved. The linear fragment ends are blunted before ligation to ablate the restriction site. Successful ligation events are then screened for the ablation of the correct restriction site. The probability of the desired site having been cleaved is inversely proportional to the total number of the target restriction sites. Conventional plasmid restriction enzyme digests were set up.

Digestion was ceased at regular intervals between 1-30 minutes with the addition of 2µl of 0.5M EDTA, pH 8, and incubation on ice. The samples were all run on an agarose gel alongside a sample of linearised DNA for accurate sizing. The linear fragments from each of the partial digests were cut out of the gel and extracted before the appropriate blunting procedure was carried out followed by ligations of the blunted fragments before transformation into bacteria and screening for the loss of the desired restriction site.

2.1.6.3. Blunting of Overhangs

When a restriction site needed to be ablated, or a subcloning step using incompatible restriction sites was carried out, unwanted overhangs created by restriction enzyme cleavage needed to be ablated. For blunting 5' overhangs, the Klenow fragment of *E.coli* DNA polymerase I (Roche) was used. The Klenow fragment possesses strong 5'-3' polymerise activity and 3'-5' exonuclease activity but lacks the 5'-3' exonuclease activity of DNA polymerase I. For each µg of DNA in the reaction 2U of Klenow enzyme were added – a 2-fold excess to what is optimal for the incorporation of sufficient nucleotides to fill in a restriction enzyme overhang. The enzyme was added directly to the blunting reaction with 200µM dNTPs (Invitrogen™). A high concentration of dNTPs was added as a precautionary measure to prevent the possibility of the exonuclease activity being stimulated by a lack of nucleotides for the polymerase activity to predominate. Reactions were carried out at room temperature for 30 minutes. The enzyme was inactivated at 75°C for 15 minutes before phenol:chloroform extraction and ethanol precipitation were carried out to ensure the removal of the enzyme from the DNA solution.

For 3' overhangs, bacteriophage T4 DNA polymerase (Roche) was used. Like Klenow enzyme, T4 DNA polymerase has a 5'-3' polymerase activity and a 3'-5' exonuclease activity, however, T4 DNA polymerase's exonuclease activity is approximately 200 times stronger than Klenow's and is ideal for the removal of 3' overhangs. The enzyme was provided with a 5x reaction buffer to be used at a 1x final concentration with the enzyme. The restriction enzyme digests were first

purified with phenol:chloroform and an ethanol precipitation. The reaction also required the addition of 33μM of each dNTP (final concentration). This was done to reduce the extremely strong exonuclease activity that would otherwise have the potential to degrade the DNA beyond the overhang. 4U of enzyme (2U/μl) were used for blunting 0.5μg of DNA with 3'overhangs. The reaction was incubated for 1 hour in a 12°C water bath. The enzyme was removed by purification of the DNA from the reaction as with the Klenow enzyme.

2.1.6.4. Dephosphorylation of DNA Ends

In subcloning steps when the vector receiving a DNA fragment is cleaved at a single site, or at two different sites that are subsequently blunted, the overhangs produced are always dephosphorylated prior to a ligation step being varied out. This is to reduce the likelihood of the compatible overhangs or blunt ends of the vector religating rather than ligating with the fragment added for insertion into the open site of the vector because at least one phosphate group on DNA ends is required in a ligation step to form a phosphodiester bond to link the nucleotides together. If the phosphate groups are missing from both ends of the linear vector, the vector can no longer religate but can still accept and ligate with the insert, which has not been dephosphorylated. To carry out this dephosphorylation step, calf intestinal alkaline phosphatase (CIP) (New England Biolabs® Inc.) was used. Alkaline phosphatase catalyses the removal of 5' phosphate groups from nucleic acids. The enzyme was supplied with an appropriate 10x reaction buffer. The DNA had to be suspended in 1x buffer at a final concentration of 0.5μg/10μl. The reaction was supplemented with 100μg/ml bovine serum albumen (BSA) to enhance the efficiency of the reaction. For each μg of vector DNA being dephosphorylated, 0.5 units of CIP were added to the reaction mix. The reaction mix was incubated at 37°C for 1 hour. CIP cannot be heat inactivated. To remove the enzyme, a phenol:chloroform extraction followed by an ethanol precipitation was carried out.

2.1.6.5. DNA Ligations

To link linear strands of DNA in subcloning steps, T4 DNA ligase was used in the Rapid DNA Ligation Kit (Roche). T4 DNA ligase catalyses the formation of phosphodiester bonds between compatible 3'hydroxyl and 5'phosphate termini of linear DNA. The vector and insert DNA were combined in a total volume of 9µl if possible. For simple ligation steps, a 5:1 insert:vector molar ratio was used for optimal ligation efficiency. In cases where the insert was close to the size of the vector or even larger than the vector, a 10:1 or 20:1 insert:vector ratio was used to compensate for a lowered insertion frequency of larger DNA fragments. Typically 100-200ng total DNA concentrations were used in ligation reactions depending on the volume and concentration of DNA available for use in the ligations. To the 9µl DNA mix, 10µl of 2x ligation buffer provided with the kit was added and mixed. 1µl of T4 DNA ligase (5U/µl) was added to the mixture, which was incubated at room temperature (15°C-25°C) for at least 5 minutes. If the DNA being used for ligation was not very concentrated and the 20µl reaction size had to be scaled up, the reaction was allowed to proceed for 30 minutes to allow for the increase in volume. For each ligation carried out an insert and a vector control was also carried out where the same concentration of each of the DNA components was placed in a ligation reaction on its own. This was to assess what proportion of ligation events in the reaction were due to religated vector or contamination from uncut vector and the uncut insert containing plasmid.

2.1.6.6. Transformation of Ligation Reactions

Ligation reactions were screened for success by transformation into XL10-GOLD® Ultracompetent cells (Stratagene) (Genotype:Tet^r D*(mcrA)183* D*(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F¢ *proAB lacI* ^{q}*ZDM15* Tn10 (Tet^r) Amy Cam^r]). Ultracompetent cells have far higher transformation efficiencies than the DH5α™ competent cells used for easy transformations. As successful ligations in subcloning steps were highly inefficient, by using bacterial cells that were more likely to accept plasmids, the likelihood of isolating a successful ligation event was increased. One vial of XL10-GOLD® Ultracompetent cells was thawed on ice. The cells were mixed by flicking the tube gently. 100µl of the cells

were pipetted into prechilled 1.5ml microcentrifuge tubes for each transformation reaction to be carried out. 4µl of β-mercaptoethanol (concentration not given) supplied with the cells, were added to the cells and mixed by pipetting. The cells were placed on ice for 10 minutes and swirled gently every 2 minutes. Between 0.1 and 50ng of ligation mix in a maximum volume of 2µl were added to the cells and mixed. The tubes were incubated on ice for 30 minutes. The cells were heatshocked for 30 seconds in a 42°C water bath and placed on ice for 2 minutes to recover. 900µl of LB medium were added to the tubes, which were then incubated for 1 hour in a 37°C water bath. 100µl of the cell suspension were plated on LB agar plates with the appropriate selection. The remaining 900µl were spun in a microcentrifuge at 4000*g* [7000rpm] for 3 minutes. All but 50µl of the supernatant were removed. The pellet was resuspended in the remaining supernatant and plated on the appropriate LB agar plates as well, in case the 100µl plated for each sample did not yield many colonies. The plates were incubated overnight at 37°C. The following day, if colonies were present on the ligation plates, they were picked, expanded in LB cultures and lysed for plasmid DNA that were analysed by restriction enzyme digestion to see if the subcloning step had been successful.

2.1.7. Extraction and Analysis of RNA

2.1.7.1. RNA Isolation

In order to isolate total RNA from tissues and cells, the RNeasy® Mini kit (QIAGEN®) was used. Attempts were made to use more simple extraction methods however this kit provided the most consistent results with respect to low RNA degradation and high yield. The kit works using steps to lyse the cells followed by denaturation in guanidine isothiocyanate, which inactivates any present RNases, the RNA samples were bound to a silica gel membrane before any DNA (the silica membrane has been optimised against DNA binding), and other cellular contaminants were washed through the column before RNA elution.

For each 10^7 cells to be lysed, 600µl of Lysis buffer RLT (guanidine thiocyanate based, exact components not given) were added to the cells – either directly if the

cells were growing as a monolayer, or to a cell pellet if the cells were in suspension. The cells were pipetted to ensure complete lysis and applied to a QIAGEN® QIAshredder column. The column was spun for 5 minutes at 13000*g* [13000rpm] in a microcentrifuge in order to homogenise the sample and shear the genomic DNA present in the cell lysate. To the homogenised lysate, an equal volume of 70% ethanol, made up in diethyl pyrocarbonate (DEPC) treated water (to remove any RNases) was added and mixed with pipetting. The sample was applied to an RNeasy mini column placed in a 2ml collection tube and spun at 8000*g* [10000rpm] for 15 seconds. The flowthrough was discarded and the process repeated if the entire sample could not be deposited in the column with a single application. 700µl of buffer RW1 (ethanol based, exact components not given) were added to wash the column. The column was spun again at 8000*g* [10000rpm] for 15 seconds. The flowthrough was discarded again. Next, 500µl of buffer RPE was added to the column. The column was spun at 8000*g* [10000rpm] for 2 minutes. The RNeasy column was carefully transferred to a fresh 2ml collection tube avoiding any contact with the buffer. To elute RNA, 50µl of RNase-free water were applied to the silica in the column. The tube was spun for 1 minute at 8000*g* [10000rpm]. The eluate was pipetted back onto the column and centrifuged through a second time to ensure the greatest yield possible. RNA samples were quantified using a spectrophotometer and stored at –80°C to ensure minimal RNase activity.

2.1.7.2. Complementary DNA Synthesis

Before complementary DNA synthesis, RNA samples were treated with DNase to eliminate any contamination by genomic DNA that could affect reverse transcriptase PCR (RT-PCR) accuracy and sensitivity. To 15µl RNA, 1.8µl of 10x DNase buffer and 1µl DNase I (Promega) were added in an RNase-free 1.5ml tube. The reaction was incubated at room temperature for 15 minutes. 2µl of Stop Solution (10x) supplied with the enzyme were mixed in with the reaction, which was then incubated at 65°C for 10 minutes after which the tube was put on ice.

The SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen™) was used for the synthesis of cDNA. All the following reagents used in the synthesis of

cDNA were part of the kit and purchased from Invitrogen™. To the DNase-treated RNA, 2µl of 10mM dNTP mix and 2µl oligo(dT) mix (0.5µg/µl) were added. The tube was incubated at 65°C for 10 minutes and placed on ice for 1 minute. To this reaction, 4µl of 10x RT buffer was added, 8µl $MgCl_2$ (25mM), 4µl 0.1M dithiothreitol (DTT) and 2µl RNaseOUT™ Recombinant RNase Inhibitor were added. The tube was incubated at 42°C for 2 minutes. To each tube, 2µl (50U/µl) of SuperScript™ II reverse transcriptase (RT) were added and mixed well. The tubes were maintained at 42°C for 1 hour. The reactions were heat-inactivated by incubation at 70°C for 15 minutes and then chilling on ice. For each reaction, an RT control was carried out. All the reagents for the reaction were included except the RT. This checked for remaining DNA contaminants in the reaction that would go undetected past the cDNA synthesis stage and which could give false positive RT-PCR results. For each set of reactions carried out at the same time, a reagent control was also carried out. This involved the replacement of the initial RNA sample with RNase-free DEPC-treated water. This checked the reagents in the kit for contaminating RNA or DNA that could contaminate the RNA samples being used with the kit and could affect the cDNAs synthesised and the RT-PCR accuracy.

If the reactions were scaled up in volume, the addition of reaction reagents was scaled up correspondingly as were the incubation times.

2.1.8. Polymerase Chain Reaction

2.1.8.1. Primer Design

In the design of primers, several parameters needed consideration. Through convention, PCR oligonucleotides were designed to be approximately 20 nucleotides in length. The oligonucleotides needed to have similar T_ms (melting temperatures) to other primers intended for use with each other so that the reaction conditions suited both primers in the annealing step of a PCR reaction. The T_m was ideally below 70°C to allow the annealing and extension steps to work effectively. A region of sequence that was not too G/C rich was preferential as the annealing step would be more efficient and the T_m of the primer was likely to be lower. Primers used together were checked for any opportunity for dimerisation or the formation of other

secondary structures as these could easily result in the failure of the PCR reaction, as the primers would anneal with each other and not the DNA template. Primers were obtained from MWG Biotech.

2.1.8.2. Optimisation

Platinum® *Taq* (Invitrogen™) or BIOTAQ (BIOLINE) were used for all PCR reactions carried out. To optimise the PCR reaction, a range of annealing temperatures needed to be tested. The optimal annealing temperature is usually several degrees below the lowest primer T_m of the primers being used in the reaction, although on occasion, it can be the same as the T_m of the primers. Usually, optimisation of the annealing temperature was sufficient to obtain a stringent PCR protocol and the addition of magnesium chloride ($MgCl_2$) was maintained at 1.5mM. However, if the adjustment of the annealing temperature was insufficient to propagate the PCR product alone, a reaction buffer without the addition of $MgCl_2$ (200mM Tris-HCl pH 8.4, 0.5M potassium chloride (KCl)) was used and different concentrations of $MgCl_2$ (between 1mM-4mM) added in optimisation reactions to ascertain what reaction conditions gave the best reaction stringency and amplification of the desired band. By trying a combination of annealing temperatures and supplemented Mg^{2+} ions, most PCR reactions could be optimised to give high specificity for amplification of the desired band with low to no background. If background could not be eliminated with reaction optimisation, the possibility that the primers were not behaving as they should have done was addressed and new ones needed to be considered. Reactions were carried out in a thermal cycler with the provision of a gradient block enabling the optimisation of the annealing temperature in a single PCR reaction (DYAD™ Peltier Thermal Cycler).

Gene	Primer Sequences	Annealing Temp °C	Product Size
β-actin	Forward 5'-GATGACGATATCGCTGCGCTG-3' Reverse 5'-GTACGACCAGAGGCATACAGG-3'	61	455
brachyury	Forward 5'-ATGCCAAAGAAAGAAACGAC-3' Reverse 5'-AGAGGCTGTAGAACATGATT-3'	55	815
flk-1	Forward 5'-CACCTGGCACTCTCCACCTTC-3' Reverse 5'-GATTTCATCCCACTACCGAAAG-3'	61	239
flt-1	Forward 5'-CCAAGGCCTCCATGAAGATAG-3' Reverse 5'-CTGTCAGGGGCTGGTTGTC-3'	61	247
gfp	Forward 5'-GACGTAAACGGCCACAAGTTC-3' Reverse 5'-GAAGTCGTGCTGCTTCATGTG-3'	61	167
hprt	Forward 5'-CAAGCTTGCTGGTGAAAAGGAC-3' Reverse 5'-CTTGCGCTCATCTTAGGCTTTG-3'	61	186
neo	Forward 5'-CATGATTGAACAAGATGG-3' Reverse 5'TCAGAAGAACTCGTCAAG-3'	53	800
oct4	Forward 5'-GGCGTTCTCTTTGGAAAGGTGTTC-3' Reverse 5'-CTCGAACCACATCCTTCTCT-3'	55	302
pecam1 (CD31)	Forward 5'-AGGGGACCAGCTGCACATTAGG-3' Reverse 5'-AGGCCGCTTCTCTTGACCACTT-3'	61	452
scl/tal-1	Forward 5'-GCACACACGGGATTCTG-3' Reverse 5'-GAATTCAGGGTCTTCCTTAG-3'	58	321

Table 2.1 *PCR primers used and their optimised annealing temperatures.*

2.1.8.3. PCR Procedure

A typical PCR reaction mix would be as follows:

5µl 10x PCR buffer $-Mg^{2+}$ (Invitrogen™)

1.5µl 50mM $MgCl_2$ (final concentration 1.5mM) (Invitrogen™)

0.5µl 10mM dNTPs (Invitrogen™)

0.5µl Forward Primer (1ρM final concentration)

0.5µl Reverse Primer (1ρM final concentration)

1-2µl DNA/cDNA template

0.4µl Platinum® *Taq* DNA Polymerase (Invitrogen™)

To 50µl with ddH_2O

Typical Reaction Conditions:

1. 3 minutes 95°C denaturation
2. 30 seconds 94°C denaturation
3. 30 seconds 60°C annealing
4. 45 seconds 72°C extension
5. Repeat steps 2 to 4 for 30 cycles
6. 10 minutes 72°C extension
7. Maintain samples at 10°C thereafter

10µl of the reaction were run on a 2-4% agarose gel to check the success of amplification.

2.1.8.4. RT-PCR Analysis

RT-PCR reactions were carried out as with PCR reactions. However, to ensure no contamination with genomic DNA and to normalise the concentration of template used in subsequent PCR reactions, a PCR reaction of a housekeeping gene, typically β-actin was carried out. A positive band of the correct size was expected in the cDNA synthesis reactions that included RT whereas no band would be expected in reactions that had omitted RT, thus showing a pure cDNA sample. The band

intensities from the β-actin reaction served to quantify the cDNA samples so that equivalent starting concentrations could be used in comparative PCR reactions.

2.2. Cell Culture Methods

2.2.1. Culture of Murine Embryonic Stem Cells

2.2.1.1. Complete Media

The optimal medium used for the routine maintenance of all murine ES (mES) cells consisted of Glasgow's Modified Eagle's Medium (GMEM)(Sigma) supplemented with: 5% (v/v) Foetal Calf Serum (FCS) (Globepharm), 5% (v/v) Newborn Calf Serum (NBCS) (Sigma), 2mM L-glutamine (Invitrogen™), 0.1mM non-essential amino acids (Invitrogen™), 1mM sodium pyruvate (Invitrogen™), 0.1mM β-mercaptoethanol (Invitrogen™), 500U/ml Leukaemia Inhibitory Factor (LIF) (LIF-ESGRO) (Chemicon).

All the components to be added to the GMEM were placed in a sterile bottle and mixed. Using a vacuum pump and a 0.2μm bottle-top filter unit (VacuCap® 60, Gelman Laboratory), the components were filtered into the GMEM bottle. The bottle was clearly labelled with the medium type and the date before being stored at 4°C. The components of the medium were only stable up to 4 weeks after addition to the medium (L-glutamine converted to glutamic acid after 4 weeks) so if the bottle had not been used after this period of time had elapsed, it was discarded and a fresh bottle of medium made up.

2.2.1.2. Passaging mES Cells

When a flask of cells was confluent or sub-confluent i.e. covering the flask fully or almost fully, the cells required passaging. A medium change was carried out 1.5-2 hours prior to passaging to prime the cells for the procedure and induce growth in them. The flask was washed with sterile Dulbecco 'A' PBS (no magnesium or calcium ions) (0.15M NaCl, 0.003M KCl, 0.008M disodium hydrogen orthophosphate (Na_2HPO_4), 0.001M potassium dihydrogen orthophosphate (KH_2PO_4)) (100x tablets purchased from Oxoid). To a 25cm^2 flask, 1ml of TEG (Trypsin/EGTA (TEG) solution (107.8mM NaCl, 0.845mM Na_2HPO_4, 1.59mM KH_2PO_4, 4.47mM KCl, 5mM D-(+)glucose, 22.29mM Tris, 0.0009% (w/v) phenol

red, 0.25% (v/v) trypsin (Invitrogen™), 1.05mM ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 0.000105% (w/v) polyvinyl alcohol)) was added and the flask placed in a 37°C incubator for 1-2 minutes for the trypsin to break intercellular protein bonds between the cells. The trypsinised flask was knocked several times to displace the cells from the gelatin and to break up the cell aggregates to a single cell suspension. The TEG was inactivated with serum by the addition of 5ml of ES cell medium to the flask. The cells in the medium were pipetted several times to attain a good single cell suspension before being transferred to a 15ml tube and being spun in a centrifuge (Jouan B4) at 166*g* [1000rpm] for 5 minutes. Most of the medium was removed from the cell pellet. The pellet was resuspended in the remaining medium by agitation. 5ml of fresh medium were added to the resuspended pellet which was mixed by pipetting. If the cells were being expanded, the resuspended pellet was transferred to a gelatinised 75cm^2 in 15-20ml of medium. If the cells were being maintained or seeded at a low density, a fraction of the resuspended medium was transferred to a fresh gelatinised flask with an appropriate volume of medium.

2.2.1.3. Freezing of mES Cells

To freeze flasks of cells in vials, a medium change was carried out on the flask 1.5-2 hours prior to freezing to induce growth in the cells. The cells were trypsinised in the conventional manner and spun down to form a pellet. The cells were resuspended in 50% (v/v) ES cell medium and 50% (v/v) 2x freezing mix (60% (v/v) ES cell medium, 20% (v/v) FCS, 20% (v/v) dimethyl sulfoxide (DMSO) and placed in prechilled cryovials. The vials were placed at –80°C overnight and then transferred to –150°C for indefinite storage. The number of vials that the flasks were frozen in depended on the size of the flask and the confluence of the cells: confluent 25cm^2 flasks were frozen to 1 vial, 75cm^2 flasks to 3 vials and 150cm^2 flasks to 9 vials.

2.2.1.4. Thawing of Cells

ES cells were kept frozen at -150°C. They require rapid thawing as they were frozen with DMSO, a cryopreservant to which they are sensitive once thawed. A vial of

frozen cells was removed from a –150°C freezer and immediately immersed in a beaker of 37°C water. Once thawed, small volumes of the vial were gradually added to 5ml of complete ES cell medium in a 15ml screw-capped tube. The tube was spun at 166*g* [1000rpm] for 5 minutes. All but a trace of the medium was aspirated off with a Pasteur pipette. The cell pellet was dispersed by flicking the tube. 5ml of medium was added to the resuspended pellet. The medium was pipetted several times before being added directly to a gelatinised 25cm^2 tissue culture grade flask (Nunc). The flask was marked with the cell type, date of thawing and the passage number of the cells before being placed in a 37°C humidified incubator with 5% CO_2. If filtered caps were used, they were tightly screwed onto the flasks, however, if vented caps were used, they was left loose to ensure good circulation of CO_2 in the flask.

Cells frozen in suspension in multiwell plates were thawed in a similar way. Warmed medium was added to the wells immediately after the plates were removed from cold storage. As soon as the media in the wells had thawed, the contents of each well were transferred to wells in new gelatinised multiwell plates that had wells with a larger surface area. Care was taken to replicate the order and labelling of the wells. Once all the cells had been transferred, the wells were filled with fresh ES cell medium and incubated at 37°C for 5 hours to attach to the gelatinised surface before being given a medium change to remove the DMSO cryopreservant that was still present after they had been thawed.

When multiwell plates with cells frozen as a monolayer had to be thawed, the plates were removed from –80°C storage as quickly as possible. Warmed (37°C) medium was added to the sides of the wells as quickly as possible after removal from –80°C. This medium was then aspirated off to remove the now-thawed freezing mix and fresh medium was added to the wells. Thawing of plates was best done in the morning so that a second medium change could be carried out in the late afternoon as well.

2.2.1.5. Counting Cells

When cell numbers needed to be calculated, a haemocytometer (Assistent) was used. Cells were trypsinised, pelleted and resuspended in 5ml medium. Using a Pasteur pipette, some cell suspension was taken up and dropped under either side of the haemocytometer coverslip. The 4 corners of the haemocytometer consisting of 16 square grids were counted and the mean average taken of the 4 counts. This count multiplied by 10^4 represented the number of cells present in 1ml of the cell suspension.

2.2.1.6. Counting Viable Cells

When a low cell number needed to be seeded or if the method of disaggregation was particularly harsh, cells being counted were also checked for viability. 50µl of the cell suspension being counted was mixed with an equal volume of 0.4% (w/v) Trypan Blue Solution (Sigma) and left at room temperature for 1-2 minutes. This mixture was applied to a haemocytometer. Viable cells appeared white and were counted whereas dead cells took up the blue stain and appeared blue and were ignored, in order to get an accurate estimate of the number of live cells present. The counting was carried out as described before except that the 1:1 dilution with the stain was accounted for by doubling the cell number to get an accurate cell count.

2.2.1.7. Karyotyping of mES Cells

A confluent 25cm^2 flask of ES cells was trypsinised and 20% of the cells were replated. The following day, 10µl/ml of culture medium of colcemid solution (KaryoMAX® Colcemid® from Invitrogen™) were added to the flask and mixed. The split was necessary to ensure the active growth and division of the cells. The colcemid acted to arrest the cells in the metaphase stage of mitosis. The cells were harvested one hour later by trypsinisation. The cells were washed in 2-3mls of PBS before being spun at 280*g* [1300rpm]. All but a trace of the PBS was removed. The pellet was resuspended in the remaining medium. 10mls of ice-cold hypotonic solution (0.65% (w/v) KCl, made fresh each time) were added to the resuspended pellet. The cells were left in this solution for 10 minutes at room temperature. The

cells were pelleted by centrifugation at 280*g* [1300rpm], the hypotonic solution was decanted off and the pellet of cells resuspended. The first fix solution (75% (v/v) methanol, 25% (v/v) acetic acid) was added carefully; drop wise, to the resuspended pellet. As the fix was added, the tube of cells was mixed slowly on a vortex mixer. The pellet was resuspended in 10mls of fix solution. The cells were pelleted as before. The cells were washed in fix solution twice more and spun again to be pelleted. They were resuspended in 1ml of fix solution.

Glass slides were cleaned in 70% (v/v) ethanol and moistened with cold water. 1-2 drops of the cells in fix solution were dropped onto a wet slide at a 45° angle with a fine-tipped Pasteur pipette, and allowed to run down the slide. For each cell line being karyotyped, 10 slides were prepared. The slides were left to air dry and viewed under a phase contrast microscope to check that a complete karyotype was present.

2.2.2. Differentiation Protocols

The standard medium used to carry out basic non-directed differentiation contained all the components of standard ES cell medium (as detailed in section 2.2.1.1) except LIF which was omitted from the medium. As LIF is the key supplement in the maintenance of pluripotency in ES cells, its absence was enough to allow the ES cells to differentiate freely to many lineages.

2.2.2.1. Generation of mES-Derived Embryoid Bodies in Suspension

EBs were generated for the differentiation of ES cells or as part of more complex or specific differentiation protocols. ES cells maintained as a monolayer were trypsinised, pelleted, resuspended in an appropriate volume of ES cell medium and counted. Unless following a defined differentiation protocol, 10^6 cells were plated in 10ml differentiation medium in bacterial grade 10cm dishes. The cells formed embryoid body aggregates by the second day of maintenance in suspension in the

absence of LIF. The length of incubation of EBs in suspension depended on their designated purpose. In order to prevent their attachment to the plastic plates, the EBs were pipetted every 2-4 days and the medium changed as required. To carry out a medium change, the EBs were collected in a 15ml tube. If the EBs were not yet cystic, they were centrifuged at 166*g* [1000rpm] for 2 minutes. If the EBs included cystic EBs, they were allowed to settle in the tube for 10 minutes. The medium was aspirated off and fresh medium added to the EBs before they were transferred to a new petri dish.

2.2.2.2. Generation of Embryoid Bodies in Hanging Drops

To obtain EBs of a uniform size and cell density, hanging drop cultures were set up. Cells were trypsinised and counted in the usual manner. $6x10^5$ cells were resuspended in 20ml ES cell medium and pipetted repeatedly to ensure an even distribution of cells in the medium. Using a multi-channelled pipetter, 20µl drops of the cell suspension (corresponding to a cell count of 600) were placed on the inside of the lid to a 15cm dish. This was repeated until evenly spaced 20µl droplets of the cell suspension covered the inner surface of the lid. 10ml PBS were added to the dish to maintain moisture and the lid was flipped over onto the dish so that the drops were hanging down over the PBS and incubated at 37°C. After 2 days of maintenance as droplets, the ES cells formed tight aggregates of ES cells. These were then washed into basic differentiation medium in non-tissue culture treated 10cm petri dishes and treated as the suspension cultures were.

2.2.3. Haematopoietic Differentiation

Haematopoietic differentiation was carried out in a methylcellulose culture. The methylcellulose provided a 3 dimensional matrix that haematopoietic progenitors thrived in and provided the easiest way to score and identify different haematopoietic cell types which are frequently non-adherent to growth substrates. The protocol used was devised by Dr Lesley Forrester and adapted from Wiles and Keller 1991.

The methylcellulose growth medium consisted of 1% (v/v) methylcellulose (3% (v/v) ES-Cult® methylcellulose base medium, Stem Cell Technologies), 10% (v/v)

FCS which has been verified for its ability to support haematopoietic differentiation (Anonymous2004), 340μM monothioglycerol (MTG)(Sigma), 1U/ml erythropoietin (Roche), 10μg/ml insulin, 100μg/ml murine interleukin 3 (IL3), 2mM L-glutamine (Invitrogen™) all made up in Iscove's modified Dulbecco's medium (IMDM)(Sigma). The components were combined and shaken to mix. The methylcellulose was left to settle for 10 minutes. 2-3ml of the methylcellulose mix were pipetted into 3.5cm non-tissue culture treated dishes and placed at 37°C until required. The cells or EBs to be plated were disaggregated and counted. The appropriate number of cells to plate in each dish was calculated empirically for each cell line used as there was much variation observed between different ES cell lines. The plating efficiency of a robust ES cell line was approximately 10% and a colony count of 100/3.5cm plate was optimal. Cells were therefore usually seeded at around 1000 cells per plate.

2.2.3.1. Dispase Disaggregation of Embryoid Bodies

Dispase was used to dissociate EBs for protocols that required the growth of disaggregated EB cells in methylcellulose. It was believed to be a gentler method of dissociation than the trypsin based TEG used otherwise and gave better plating efficiencies and differentiation potential (see Chapter 4 for experimental details of its use).

EBs were collected from suspension by either centrifugation or allowing the EBs to settle by the action of gravity. The EBs were washed once in PBS and suspended in 1ml PBS or IMDM and 1ml dispase (Roche). 140μg of DNase (Sigma) was added to the dispase mix to break down any DNA from cell lysis that would otherwise prevent the attainment of a good single cell suspension of EB cells. The cells were incubated at 37°C with gentle agitation for one hour after which time the EBs were broken up by gentle pipetting of the EBs. 5ml of differentiation medium were added to the cells, which were left to recover at room temperature for 5-10 minutes. The cells were pelleted by centrifugation at 166*g* [1000rpm] and washed once in PBS. They were resuspended in an appropriate volume of differentiation medium or Iscove's modified Dulbecco's medium (IMDM) to be counted.

2.2.3.2. Scoring of Haematopoietic Colonies

Haematopoietic colonies were identified based on their phenotypic characteristics as described in the Stem Cell Technologies procedure manual (http://www.stemcell.com/technical/28415_esmanual%20H.pdf) for colony assays of murine cells using methylcellulose-based medium.

2.2.4. Directed Differentiation Enriching for *flk-1* Expression

2.2.4.1. Differentiation on Collagen IV

Nishikawa *et al.*, 1998, devised this protocol. ES cells were trypsinised, Trypan Blue stained for viability and counted. 10^4 cells were plated in each well of 6-well plates coated in Collagen IV (BIOCOAT®, BD Biosciences). The cells were grown in Alpha modified minimum essential Eagle's medium (α-MEM) (Sigma) supplemented with 10% (v/v) FCS, 2mM L-glutamine and 0.05mM β–mercaptoethanol. If growth factors were added to the differentiation medium, 50ng/ml of VEGF was added to the growth medium. To select for HPRT-expressing cells, the growth medium was supplemented with HAT. Care was taken at the time of plating to mix the cells well with the medium in each well to ensure an even distribution of the cells being plated.

2.2.4.2. Differentiation in Methylcellulose: The BL-CFC Culture

This protocol was evolved over many years by Gordon Keller's laboratory for the optimal generation of the BL-CFC: the suggested *in vitro* equivalent of the haemangioblast (Wiles & Keller, 1991; Keller *et al.*, 1993; Kabrun *et al.*, 1997; Kennedy *et al.*, 1997; Choi *et al.*, 1998; Schuh *et al.*, 1999; Faloon *et al.*, 2000). ES cells were maintained in DMEM supplemented with 15% (v/v) FCS (Globepharm), 0.15mM monothioglycerol (MTG) (Sigma), 2mM L-glutamine (Invitrogen™) and 5000U/ml LIF (LIF-ESGRO) (Invitrogen™) for at least two passages. Embryoid bodies were generated in suspension by plating 4.5 x 10^3 cells/ml in IMDM

supplemented with 15% (v/v) FCS, 0.45mM MTG, 50μg/ml ascorbic acid (Sigma), 200μg/ml iron saturated (holo-) transferrin (Sigma), 10μg/ml insulin (Sigma) in 10cm bacterial grade dishes.

Embryoid bodies were collected at precise time-points ranging from 2 to 4 days after generation (See Chapter 4 for experimental details) and disaggregated using either trypsin or dispase as disaggregation enzymes. Cells were tested for viability and counted. Cells were plated in a 1% (v/v) final concentration of methylcellulose in IMDM (3% (v/v) ES-Cult™ methylcellulose base medium, Stem Cell Technologies) supplemented with 15% (v/v) FCS which has been verified for its ability to support haematopoietic differentiation, 2mM L-glutamine (Invitrogen™), 0.45mM MTG, 25μg/ml ascorbic acid (Sigma), 200μg/ml iron-saturated (holo-) tranferrin (Sigma), 5ng/ml VEGF (Sigma), 100ng/ml Stem Cell Factor (Sigma) and 25% (v/v) conditioned medium from the D4T endothelial cell line (Kennedy *et al.*, 1997) (See 2.2.4.2.1 for details of D4T maintenance and conditioning). The number of cells plated varied between 650 and 10^4 per 3.5 cm plate. 2-3ml of the methylcellulose mix were pipetted into low-adherence 3.5 cm dishes (Anonymous2004) ensuring an even coverage. The cell suspension to be plated was pipetted into the methylcellulose with a micropipetter and mixed vigorously to ensure as even a cell density across the plate as was possible. 3.5 cm dishes were incubated within 15 cm dished with an open reservoir of PBS to ensure a highly humidified environment. Methylcellulose cultures were incubated as with all other forms of tissue culture at 37°C in 5% CO_2. The methylcellulose cultures were checked regularly for the growth and development of haematopoietic and haemangioblastic colonies.

2.2.4.2.1. Producing Conditioned Medium from the D4T Endothelial Cell Line

The D4T endothelial cell line is an ES cell-derived endothelial cell line transformed with the polyoma middle T oncogene (Williams *et al.*, 1988). It was used to condition medium for haemangioblastic differentiation (Kennedy *et al.*, 1997; Choi *et al.*, 1998) was maintained on gelatinised tissue culture flasks in IMDM supplemented with 5% (v/v) FCS (Globepharm), 2mM L-glutamine (Invitrogen™) and 50μg/ml of endothelial cell growth supplement (ECGS). Cells were fed every 2-

3 days. For conditioning purposes, cells were grown to confluence and conditioning was carried out for between 1-3 days in the maintenance medium with or without the added ECGS. The medium obtained was filtered before use in the BL-CFC cultures.

2.2.4.2.2. Testing the Potentiality of Possible Haemangioblastic Colonies

After 4-6 days in the BL-CFC culture, potential BL-CFCs were identified (Kennedy *et al.*, 1997; Faloon *et al.*, 2000) and picked out of the methylcellulose cultures and plated on Matrigel™ coated 12 well plates in IMDM supplemented with 10% (v/v) FCS, 10% (v/v) horse serum, 2mM L-glutamine (Invitrogen™), 0.45mM MTG, 5ng/ml VEGF (Sigma), 2U/ml erythropoietin (Roche), 10ng/ml bFGF (Sigma), 50ng/ml IL-3 (Sigma) and 100ng/ml granulocyte-colony stimulating factor (G-CSF) (Sigma).

Colonies were cultured for 5 days. At this time, the non-adherent cells in the culture were removed. These cells were viability stained, counted and plated in a basic haematopoietic culture (as described in 2.2.4) and assessed for their ability to differentiate to form the various haematopoietic colonies.

The adherent cells were cultured to confluence, fixed with 4% (w/v) paraformaldehyde and immunostained with endothelial, haemangioblastic and muscle markers to ascertain if these cells were capable of differentiation down these lineages proving the potentiality of the BL-CFCs picked.

2.2.5. Gene Targeting in mES Cells

2.2.5.1. Preparation of targeting vector DNA

150μg of the targeting vector was digested with the linearising restriction enzyme for 2-3 hours to attain as near a complete digest as possible. A small aliquot of the digest was run on a gel to see whether it had gone to completion. After confirmation of good digestion, the linearised DNA was condensed and purified with an ethanol precipitation. After the 70% ethanol wash, the DNA pellet was handled under sterile conditions in a tissue culture hood for the ethanol to be removed and the pellet to be air-dried. The pellet was resuspended in 200μl of sterile Hepes Buffered Saline (HBS) (20mM Hepes, pH 7.0; 137mM NaCl; 0.7mM Na_2HPO_4; 6mM D-(+)glucose).

2.2.5.2. Electroporation

A confluent 75cm^2 flask of mES cells was trypsinised and resuspended in 5ml medium in a 15ml tube. A cell count was carried out and a volume of the cell suspension corresponding to 10^7 cells was pipetted into a new 15ml tube and spun at 166*g* [1000rpm] for 5 minutes to pellet the cells. The medium was aspirated off the pellet carefully with a Pasteur pipette and the pellet resuspended in 600μl HBS. The cells and targeting vector DNA were mixed and carefully pipetted into the cavity of an electroporation cuvette (BIORAD) with an electrode gap of 0.4cm. The electroporator (BIORAD Gene Pulser™) settings used were: 800V, 3μF. The cuvette was pushed between the electrodes and electroporated. An electroporation time constant of 0.1 was desirable for ES cells. A "No DNA" control of 10^7 cells only was also carried out to assess the effect of the electroporation procedure on the viability of the cells. The cells were left for 10-15 minutes at room temperature to recover after which time the cells were mixed in with 100ml of complete ES cell medium and plated out on ten 10cm gelatinised tissue culture grade plates. 20% (160μl) of the electroporation mix for the "No DNA" control was taken and added to 20ml medium and plated out on 2 plates.

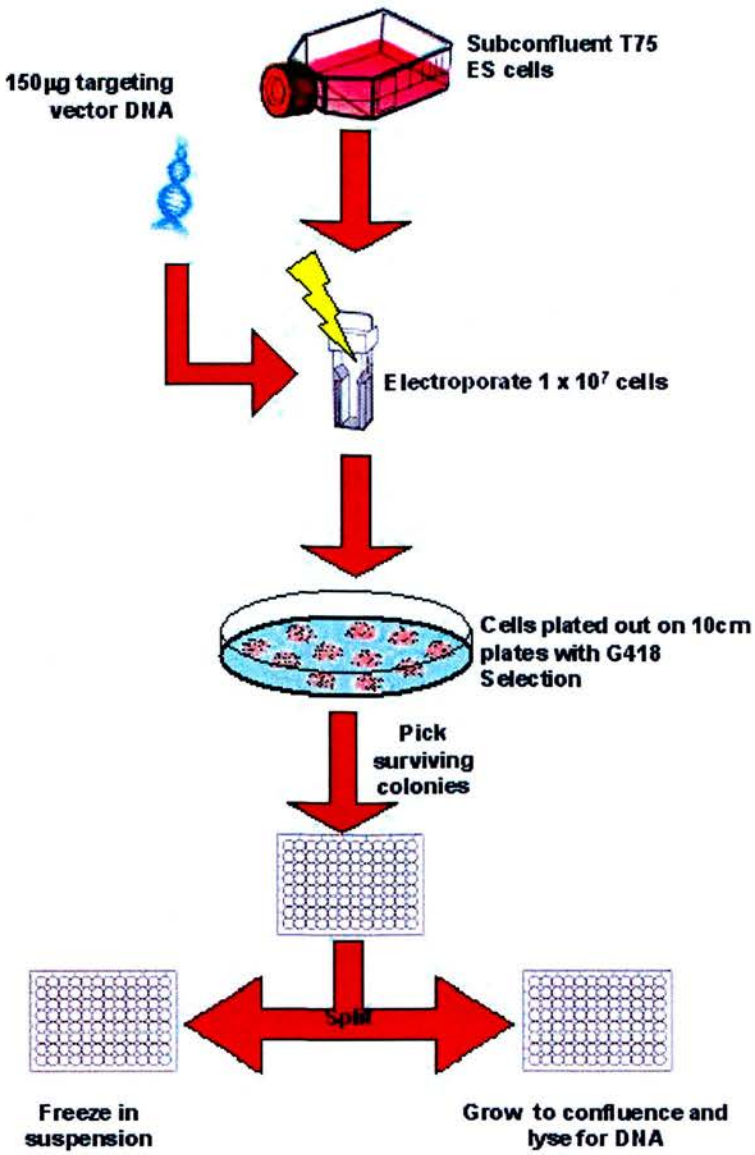

Figure 2.1 *Diagram showing the procedures involved in a gene targeting experiment.*

2.2.5.3. Selection of Potential Targeted Clones

The plates of the electroporated ES cells were maintained in complete ES cell medium for 48 hours after the electroporation. If the cells had stuck down and begun to proliferate, selection for an inserted selection cassette could be introduced to the cells via their medium. Typically, the targeting vectors possessed a PGK*neo* (phosphoglycerate kinase promoter driving the neomycin antibiotic resistance gene) cassette for selection. The neomycin analogue G418 was used for selection of cell

populations that had the PGK*neo* resistance gene integrated in their genome at a concentration of 300μg/ml in initial experiments. The level of selection was dropped to 150μg/ml because the stringency of the selection regime was excluding targeted cells from the colony pool at 300μg/ml. One targeted plate was maintained without selection to compare cell survival with the plates in selection. Also, one of the control plates electroporated without any DNA was kept without selection and one was placed in selection to assess the rate of cell death in response to the antibiotic analogue when no homologous recombination could have taken place due to the absence of any DNA. Plates were fed at least every other day until enough cell death had taken place for colonies of antibiotic resistant cells to be clearly defined and growing. Once these colonies of potentially targeted clones were visible to the naked eye, they were picked for expansion in wells for DNA (for Southern Blot analyses) and to freeze an adequate cell stock while the DNA samples were analysed to identify any targeted clones.

2.2.6. Confirmation of Targeting Events

2.2.6.1. 25cm^2 Flask Screening

Single colonies were picked with a micropipettor set to 100μl into 1.5ml microcentrifuge tubes. The colonies were disaggregated by pipetting several times after which the cells were transferred to a gelatinised well in a 24-well plate. The 24 well plates were grown to confluency. The wells were washed with 2ml PBS and trypsinised with 500μl of TEG. The cells were incubated at 37°C for 1 minute. The cells were disaggregated by repetitive pipetting and put directly into a gelatinised 6 well plate with 2ml of selection medium per well. Once the 6 well plates were confluent, the cells were trypsinised in the same manner as with the 24 well plates. This time, the trypsinised cells were split between two 25cm^2 flasks for each colony. Both were allowed to reach confluency. One was lysed for genomic DNA for Southern blots and the other vial was frozen so that if a particular colony was targeted, the corresponding vial of cells could be thawed and expanded. In a targeting experiment, the pelleting step usually carried out when cells are passaged is

omitted due to the sheer number of colonies being dealt with. If diluted out in enough serum-containing medium, TEG does not affect the viability of ES cells.

2.2.6.2. Isolation of Genomic DNA from ES Cells in Flasks

Once a flask had reached confluency, the medium was aspirated and replaced with lysis buffer (100mM Tris-HCl, pH 8.5; 5mM EDTA; 0.2% SDS; 200mM NaCl; 100µg/ml Proteinase K) (Laird *et al.*, 1991). A 25cm^2 flask required 3ml lysis buffer. The flasks were incubated overnight at room temperature with slow shaking (Luckham R100 Rotatest Shaker). The following day, an equal volume of isopropanol was added to the flask and incubated on the shaker at room temperature for 4-5 hours. The DNA from this number of cells was usually visible and was therefore removed from the flasks using a disposable inoculating loop and placed in a 1.5ml microcentrifuge tube containing 250µl of TE buffer. An ethanol precipitation procedure was carried out for the samples to remove any traces of isopropanol in the sample. After precipitation, the DNA pellet was again resuspended in 250µl of TE buffer. If the DNA concentration was very high, and resuspension in this volume was incomplete, the volume of TE was gradually increased until the viscosity of the DNA was reduced and the DNA had dissolved in the buffer.

2.2.6.3. High Throughput Screening

This method of colony processing and analysis limits the expansion of individual colonies to 96 well plates therefore it has the advantage of being far less labour intensive than the more established method of growing colonies up to two 25cm^2 flasks. The disadvantage is that not as much DNA is obtained from the lysis of a 96 well plate so there is less scope for error in the Southern analysis of the genomic DNA. There is also a higher risk of loss of a colony frozen in 96 well plate format. As before, once the colonies from cells used in a targeting experiment plated on 10cm plates became visible to the naked eye, they were picked. The colonies were rinsed with PBS and a further 10ml of PBS was added to each of the plates. The colonies were picked with a 20µl micropipetter into wells of a gelatinised 96 well plate. This was carried out until either 45 minutes had elapsed, or the 96 well plate had been filled with picked colonies. If 45 minutes passed and a plate still had

colonies to be picked, the plate was refilled with ES cell medium and left to recover while another plate was picked for 45 minutes, before PBS was reapplied to the first plate and the picking resumed. Using a multi-channelled pipetter (Finnpipette®, Labsystems), 25µl of TEG were delivered to each well on the plate and incubated at 37°C for 1 minute. 150µl of ES cell medium were then added to each well and pipetted vigorously several times to attain a single cell suspension. This was carried out until all colonies had been picked or if there were many hundreds of colonies, until 6x 96 well plates had been filled. The plates were fed the subsequent day, and every day thereafter until each single plate had attained the best overall confluency i.e. until most of the wells were confluent but before some had become over-confluent. When this point was reached, the medium was aspirated and the wells washed with 200µl PBS. This was removed and 25µl of TEG were added to each well. The 96 well plate was incubated at 37°C for 1 minute. The TEG was inactivated with the addition of 75µl of "Quench" medium (50% (v/v) ES cell medium, 50% (v/v) FCS, filtered). When the Quench medium was added, the dislodged cells were pipetted several times to disaggregate them. 50µl of the cell suspension were removed from each well and placed in another gelatinised 96 well plate. A multichannelled pipette was used and great care was taken to replicate the positions and the labelling of the wells in the plates. To each well of this plate, 100µl of ES cell medium were added and the plate was incubated at 37°C with 5% CO_2. To the original plate – also containing 50µl cell suspensions per well, 50µl of 2x freezing mix (60% (v/v) ES cell medium, 20% (v/v) DMSO, 20% (v/v) FCS, all filtered) were added. The mix was mixed in with the cell suspension by gently tapping the 4 corners of the plate. The lid to the plate was taped in place and the plate was incubated at –80°C immediately.

2.2.6.4. Isolation of Genomic DNA from ES Cells in 96 Well Plates

The plate in the incubator was fed daily and allowed to reach confluency. This plate was used to isolate genomic DNA from the ES cells so as each well reached confluency, the medium was replaced with 50µl of lysis buffer (100mM Tris-HCl, pH 8.5; 5mM EDTA; 0.2% SDS; 200mM NaCl; 100µg/ml Proteinase K) and

incubated at 37°C to allow the other wells to catch up. Once all the wells that had growing cells in them had reached confluency and they had been left to lyse for at least one overnight incubation with lysis buffer, the cell lysates were transferred to V-bottomed 96 well plates. 50µl of isopropanol were added to each well and the plates were incubated on a shaker for 10 minutes. The plates were then spun at 3220*g* [4000rpm] (Eppendorf centrifuge: 5810R) for 5 minutes to pellet the DNA. The lysis buffer was removed from the wells and the pellets were washed with 70% ethanol with great care so as not to dislodge and lose the pellet. The pellets were left to air dry for a very short period of time before they were resuspended in 50µl of TE buffer at 65°C.

2.3. 2.3 Analytical Methods

2.3.1. Southern Blotting

For confirmation of the occurrence of a targeting event in ES cells, Southern blots were carried out (Southern, 1975). This technique works by the transfer of DNA from a gel to a nylon membrane via capillary action. Genomic DNA was first extracted from ES cell colonies, which were candidates for targeting. Low volume overnight and day digests were carried out with restriction enzymes that would result in fragments that, when probed, would clarify whether targeting had taken place or not - in accordance with the designed screening strategy. The digests were loaded on a gel with a size marker e.g. Lambda/Hind III and run until the samples had migrated all the way down the gel. The gel was photographed under UV light. This was carried out to ensure that each sample loaded on the gel contained a high enough concentration of genomic DNA for the Southern to work.

The gel was first incubated in enough 0.25M HCl to cover the gel with shaking for 20-30 minutes. This was carried out to depurinate the DNA in the gel i.e. to break some purine bonds in the DNA molecules to allow better transfer of the DNA onto a blot membrane. The gel was washed in deionised water. The gel was placed in denaturation solution (1.5M NaCl; 0.5M NaOH) for two 15-minute washes. The gel was washed in water again and placed in neutralisation solution (1.5M NaCl; 0.25M Tris-HCl, pH 7.5; 1mM EDTA, pH8) for two 15-minute washes. 20x SSC (1.5M NaCl; 1.5M trisodium citrate) was poured into a basin which had a glass plate on top – bridging from one end to the other; one that was narrow enough to have gaps on either side yet large enough to hold the gel of the DNA to be blotted. A large double layer of blotting paper (Schleicher and Schuell) was bent over the glass plate and dipped, from either side of the glass plate, into the 20x SSC. Some 20x SSC was also poured over the top of the glass plate to fully wet the blotting paper. Using a glass pipette rolled over the paper, all air bubbles were removed from beneath the layers of paper. The denatured and neutralised gel was flipped onto the blotting paper – wells-side down. Any exposed blotting paper beneath the gel was carefully covered with Parafilm to prevent the bypassing of the gel when capillary transfer of

20x SSC up into the Southern setup took place. A piece of positively charged nylon membrane (Roche) was cut to the same size as the gel and wet in 2x SSC. The nylon was kept as clean as possible, for even transfer of DNA from the gel onto it, so it was handled with clean blunt forceps at all stages. It was placed on top of the gel and rolled with the pipette to eliminate air bubbles from under the membrane. A piece of blotting paper the same size as the gel was wetted in 2x SSC and placed onto the nylon membrane and rolled. On top of that, 2 sheets of dry blotting paper of the same size were added. A stack of paper towels was added to the setup so the membrane and blotting papers were completely covered. A second glass plate was placed over the stacked towels to evenly distribute the weight, and a 0.5-1kg weight was added on top. The blotting setup was left overnight. The capillary uptake of 20x SSC through the blotting paper and the gel transferred the DNA onto the nylon membrane – the 20x SSC being drawn up through the setup and soaked up in the paper towels.

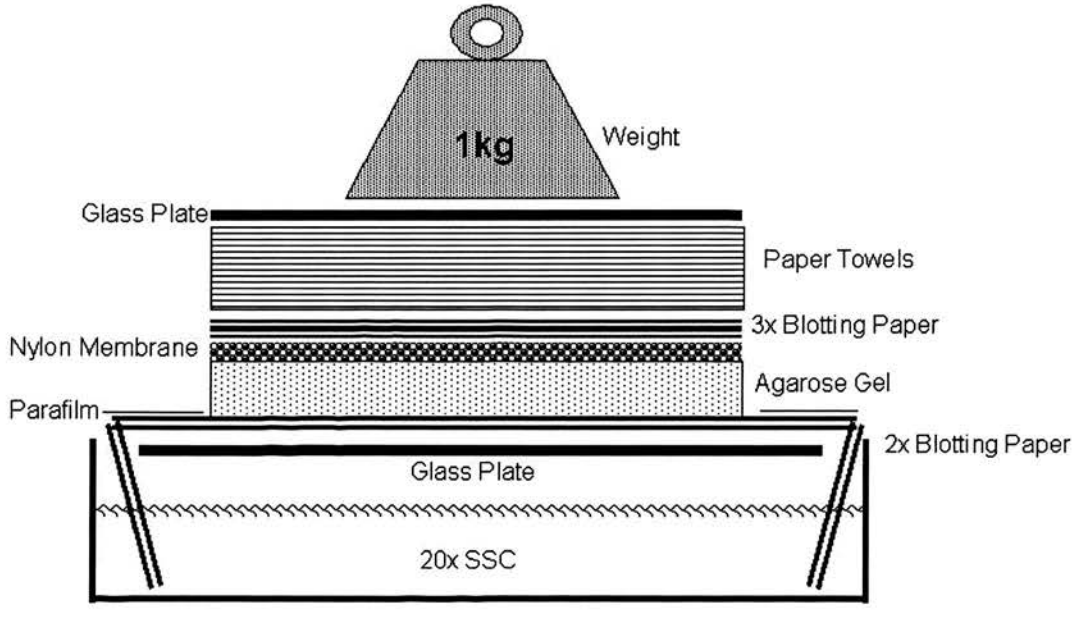

***Figure 2.2** Southern blot setup*

The next day, the blotting apparatus was carefully taken apart. The nylon membrane and gel were flipped over and the wells of the gel marked onto the membrane with a blunt pencil through the gel. The gel was stained in 1x TAE with added ethidium bromide to check that all the DNA has transferred onto the membrane. The

membrane itself was incubated in 3x SSC to reduce the salt content in the membrane and dried on blotting paper. Once dry, the blot was placed on a new piece of blotting paper and covered in Saran wrap. The DNA on the blot was crosslinked onto the membrane in a UV Stratalinker® 2400 (Stratagene®) using the autocrosslink program. The blot was subsequently hybridized with a radiolabelled probe and laid down on X-ray film to produce an autoradiograph of the probe's hybridisation.

2.3.1.1. Production of a Radiolabeled Probe

Two probes were made, one for the genomic DNA to bind to a restriction fragment that would indicate whether targeting had taken place, and a weaker radiolabeled probe for the Lambda/Hind III size marker. The probe for the genomic DNA was purified from an agarose gel and quantified.

A total concentration of 25ng of probe was required for the labeling reaction. 25ng of the Lambda/Hind III marker was also used for labelling. The Rediprime™ II random prime labelling system (Amersham Pharmacia Biosciences) was used for the labelling reaction. The protocol is based on one devised by Feinberg and Vogelstein (1983; 1984) where random hexanucleotides are used to prime DNA synthesis off denatured DNA strands using Klenow enzyme's 5'- 3' polymerase activity. The use of radioactive nucleotides in this reaction enables a radiolabelled DNA probe to be made, which on denaturation, can bind to DNA on the nylon membrane. This kit was used in conjunction with Redivue™ $\alpha[^{32}P]$ dCTP (Amersham Pharmacia Biosciences). The probe DNA was diluted to 25ng/45µl in TE buffer. The double-stranded DNA was denatured by heating in a boiling water bath for 10 minutes after which it was snap cooled by incubation on ice. The tube of probe DNA was spun briefly to draw any condensed liquid in the lid back down to the bottom of the tube. The DNA sample was added to one reaction tube of Rediprime™ mix. To the genomic DNA probe, 5µl of Redivue™ $\alpha[^{32}P]$dCTP were added and the mixture pipetted several times to resuspend the components of the Rediprime™ pellet. The priming reaction was incubated for 10 minutes in a 37°C waterbath. The Lambda/Hind III probe needed to be weaker because the multiple copies of the Lambda/Hind III fragments had a much higher binding frequency to the probe and

could have interfered with the genomic probe in the Southern blot hybridisation due to a disproportionately strong signal from the size marker. For this probe, only 1µl of Redivue™ α[^{32}P]dCTP was used. The remaining 4µl of dCTP were unlabelled nucleotides added to the reaction at a concentration of 0.5mM. The two probes were labelled identically otherwise.

A NICK™ Column (Amersham Pharmacia Biosciences) was used to purify each of the labelled probes separately from any unincorporated nucleotides. The column consisted of Sephadex® G-50 in distilled water that would separate out differently sized molecules from each other and had been optimised for the removal of unincorporated nucleotides. The column was rinsed in TE buffer once. The Sephadex® gel equilibrated with the addition of 3ml of TE buffer to the column, which was allowed to pass through the column. The labelled probe was added to the column. When the probe had entered the gel, 400µl of TE buffer were added to elute out the unincorporated nucleotides. Another 400µl of TE buffer were loaded onto the column to elute the labelled probe from the column into a 1.5ml collection tube. 1µl of the eluted probe was spotted onto a piece of fiberglass paper and placed in a scintillation bottle. The sample was quantified for radioactive emission in a scintillation counter (Wallac 1410 Liquid Scintillation Counter; programme 1-8) to ensure that the probe was radioactive enough to raise a signal once placed on film. The probe was denatured with the addition of 40µl of 5M NaOH and added to the hybridisation solution.

2.3.1.2. Hybridisation Procedure

The Southern blot was placed in a preheated (65°C) hybridisation tube with 20ml preheated hybridisation solution (0.5M Na_2HPO_4; 7% SDS; 1mM EDTA) in a 65°C oven for 1 hour prior to hybridisation. The hybridisation solution was emptied from the tube and replaced with 15ml of fresh preheated hybridisation solution and the two labelled probes. The tube was placed at 65°C with constant rolling overnight. The following day, two 15-minute washes with 20ml Wash 1 (2x SSC; 0.1% SDS) and Wash 2 (0.2% SSC; 0.1% SDS) were carried out. Excess liquid was removed from the membrane although care was taken so as not to allow it to dry out. The

membrane was wrapped in Saran Wrap and placed on X-Ray film (Kodak Biomax™ MS) in an autoradiography cassette at –80°C for 1 week to develop the autoradiograph of the Southern blot. The photographic film consists of a plastic sheet covered with a light sensitive chemical emulsion e.g. silver nitrate or silver halide that undergo a chemical change on exposure to light or any form of electromagnetic radiation. The chemical conformation can be visualised after the film is fixed and developed.

2.3.2. Immunohistochemistry

2.3.2.1. Immunostaining of Fixed Cells

Cells grown as a monolayer were first fixed before antibody staining. The growth medium was aspirated and the cells were washed once in PBS. 4% paraformaldehyde was typically used as the fixative. This chemically cross-links cellular components to each other therefore stabilising antigens in place. Cells were fixed with cold paraformaldchyde for 20 minutes at 4°C. The fixative was washed off with 4 washes of PBS. One quick rinse followed by three washes of 5-10 minutes were carried out at room temperature with gentle agitation. If the antigen being stained for was an intracellular antigen, the cells were permeabilised with a 2-minute wash with 100% ethanol. This was followed by two 5-minute rinses with PBS. If the antigen was a cell surface antigen this was not required.

Non-specific immunoglobulin binding was blocked by incubation in a 10% (v/v) serum solution diluted in PBS for 1 hour at room temperature with gentle agitation. If possible, serum from the animal in which the secondary antibody was raised in was used as the blocking agent to reduce the possibility of background from the non-specific binding of the secondary antibody. Often this was not practical and BSA (Invitrogen™) was used instead.

The primary antibody was applied directly after the blocking step. Typically, polyclonal antibodies were used for cell staining as they are believed to give a better signal intensity than monoclonal antibodies and are cheaper. In instances where

background was a problem, monoclonal antibodies were used to reduce the background staining. With each new antibody used, an initial optimisation step was carried out with different antibody dilutions to see which gave the best specificity and the least background. The antibodies were diluted in 1% (v/v) of the appropriate serum made up in PBS. Dilutions usually ranged from 1/100 to 1/1000 (v/v). The primary antibody was left on the cells overnight at 4°C with gentle agitation or at room temperature for 1 hour. The antibody was washed off the cells with four PBS washes as before.

A fluorescently tagged secondary antibody was applied at a dilution of 1/200 in 1% (v/v) serum for 1 hour at room temperature, with agitation, and washed with PBS washes as before. All steps from the point of application of a fluorescently labelled antibody were carried out under darkness (Zwaginga & Doevendans, 2003). The cells were mounted with Vectashield® mounting medium with 2-(4-amidinophenyl)-6-indolecarbamidinedihydrochloride (DAPI) (Vector Laboratories) and a glass coverslip. DAPI is a fluorochrome that binds DNA and will therefore allow the nucleus of cells to be visualised. The edges of the coverslip were sealed with PANG liquid sealant. Slides and plates were observed under a microscope with a DAPI filter and the appropriate fluorescence filter.

2.3.2.2. Preparation of Cryopreserved Tissue Sections

Tissues of interest were isolated and fixed in 4% paraformaldehyde in PBS at 4°C. The length of fixation was dependent on the density and size of the tissue. After fixation, the tissue was washed 3 times in PBS and transferred to 0.12M phosphate buffer (0.19M Na_2HPO_4, 0.046M sodium dihydrogen orthophosphate-1-hydrate ($NaH_2PO_4.H_2O$)) supplemented with 15% (w/v) sucrose overnight at 4°C. Good impregnation of the tissue with the phosphate buffer resulted in the tissue sinking in the solution. If after an overnight incubation, the tissue had not sunk to the bottom of the container, fresh phosphate buffer was added to the tissue sample and the tissue incubated at 4°C until the tissue did fully sink in the solution. The tissue was next incubated in pre-warmed 0.12M phosphate buffer supplemented with 15% (w/v) sucrose and 7.5% (w/v) gelatin for 30-60 minutes at 37°C.

In appropriately sized moulds, a cushion of 0.12M phosphate buffer supplemented with 15% (w/v) sucrose and 7.5% (w/v) gelatin was poured and left to set. The tissue was positioned on this cushion and immersed in more phosphate buffer with sucrose and gelatin. The gelatin block was allowed to set at 4°C for 30 minutes before being cut to a smaller cube encasing the tissue.

The gelatin block was fixed to a cardboard mount with embedding compound (Webb *et al.*, 2003). A beaker of isopentane was cooled to approximately –80°C in liquid nitrogen. The gelatin block was immersed in the cooled isopentane for 1 minute before being transferred to dry ice and long term storage in a –80°C freezer.

14µm thick sections were cut at –20°C with a cryostat (Bright OTF5000) and collected on Superfrost plus electrostatically charged microscope slides (BDH). The sections were stored at –20°C until use.

2.3.2.3. Immunostaining of Cryosectioned Tissue

Sections were removed from cold storage and allowed to rise to room temperature and dry out by incubation at room temperature for 1 hour. Slides were rehydrated by incubation in PBS for 5 minutes. Non-specific epitopes were blocked and the tissue sections permeabilised by incubating slides in 2% (v/v) BSA (Invitrogen™), 0.2% (v/v) polyoxyethylenesorbitan monolaurate (Tween20) in PBS for 15 minutes. Primary antibodies were diluted in the blocking solution and applied to the slides for an overnight incubation at 4°C. Following incubation with a primary antibody, sections were washed in PBS supplemented with 0.1% (v/v) Tween20, three times for 5 minutes each time. Fluorescently labelled secondary antibodies were used and all steps following the application of the secondary antibodies were carried out in the dark. The antibodies were diluted in PBS and applied to the slides for one hour at room temperature. Three washes were carried out in PBS supplemented with Tween20 as before. The slides were mounted in Vectashield® supplemented with a DAPI stain and a coverslip and sealed with PANG. Slides were stored in the dark at 4°C.

Antibody	Isotype	Working Dilution	Manufacturer
FLK-1	Mouse Monoclonal Ig G_1	1:1000	Santa Cruz Biotechnology
FLK-1	Rabbit Polyclonal IgG	1:500	Santa Cruz Biotechnology
SCL/TAL1	Goat Polyclonal IgG	1:200	Santa Cruz Biotechnology
VE-CADHERIN (Yang *et al.*, 1999)	Goat Polyclonal IgG	1:500	Santa Cruz Biotechnology
GFP	Monoclonal IgG_1, κ	1:200	Chemicon
α-SMA	Mouse monoclonal ascites fluid	1:1000	Sigma
Anti-Mouse FITC conjugate	IgG	1:200	Calbiochem
Anti-Mouse Alexa Fluor 350 conjugate	IgG	1:200	Molecular Probes
Anti-Goat Cy3 conjugate	IgG	1:200	Sigma
Anti-Rabbit FITC conjugate	IgG	1:200	Sigma

Table 2.2 *Antibodies used for immunohistochemistry and their dilutions.*

2.3.2.4. Preparation of Wax-Embedded Tissue

Tissues to be wax embedded were fixed in paraformaldehyde and washed as carried out for cryopreservation. The tissue was placed in a plastic wax embedding cassette and placed in an automated tissue processor (Shandon Hypercentre® XP) and processed through the dehydration steps shown in **Table 2.3**.

% Concentration (v/v)	Solution	Incubation Time (mins)	Temperature (°C)
70	Ethanol	90	RT
96	Ethanol	90	RT
96	Ethanol	90	RT
100	Ethanol	90	RT
100	Ethanol	90	RT
50/50	Ethanol/PCA	90	RT
50/50	Ethanol/PCA	60	RT
100	PCA	60	RT
100	PCA	60	RT
100	PCA	60	RT
100	PCA	60	RT
100	Paraffin Wax	60	60
100	Paraffin Wax	60	60

Table 2.3 *Dehydration and perfusion steps used in automated wax embedding.* ***PCA****, paraffin-clearing agent;* ***RT****, room temperature.*

The tissue was left in molten paraffin wax at 60°C for several hours before being set in a block of wax in a mould. The block of wax was sectioned using a rotary microtome (Microm HM325) and the sections affixed to polylysine coated slides ("Polysine" slides, BDH) by incubation at 55°C overnight.

2.3.2.5. Haematoxylin and Eosin Staining

Paraffin sections were de-waxed in xylene for 5 minutes. The tissues were rehydrated with a 2-minute incubation in 75% (v/v) ethanol followed by a 2-minute incubation in 65% (v/v) ethanol. The slides were immersed in water for 5 minutes. The slides were stained in haematoxylin stain (BDH) for 2 minutes. The slides were washed in a constant stream of water for 5 minutes or until the sections appeared blue. The slides were counterstained in eosin stain (BDH) for 2 minutes and washed in water for 5 minutes. The sections were dehydrated again with 1-minute

incubations in 65% ethanol followed by 1 minute in 75% ethanol before being transferred to xylene for 5 minutes. The slides were mounted in DPX (xylene (80% (v/v) based mountant containing distyrene and tricresyl phosphate) and covered with a coverslip. The slides were allowed to settle overnight before microscopy.

2.3.3. Western Blotting

Western blotting is a sensitive technique for detecting specific protein molecules using antibody conjugation after a similar blotting method as that used to detect DNA in a Southern blot. Approximately 20 femtomoles of a protein are detectable with this technique approximating to 1ng depending on the molecular weight of the protein (Harlow & Lane, 1988).

2.3.3.1. Cell Lysis

Initially, cells were lysed under denaturing conditions. This prevented solubilisation, aggregation or coprecipitation of proteins (Sambrook & Russell, 2001), which may have affected the accuracy of protein detection and quantitation. 1ml of protease inhibitor cocktail (1 tablet (Roche) in 7ml PBS) were added to the cells. This volume was suitable for up to approximately 10^7 cells. The volume added was adjusted relative to the number of cells if there were more than 10^7 cells being lysed. An equal volume of SDS lysis buffer (100mM Tris pH 6.8, 4% (w/v) SDS, 0.2% (w/v) Bromophenol Blue, 20% (v/v) glycerol, 200mM dithiotheitol (DTT)(added fresh to the other buffer ingredients each time)) was added to the cells. The cells were scraped off the growth surface if they had been growing as a monolayer or pipetted several times with a micropipettor if they had been growing in suspension, to ensure good lysis.

The lysates were then loaded onto QIAGEN® QIAshredder™ columns and spun at 13000*g* [13,000 rpm] for 5 minutes to disrupt genomic DNA in the lysates. At this point, samples were aliquoted into single use aliquots and stored at –80°C. If used immediately, or once thawed from the stocks, the samples were boiled for 5 minutes, either in a 100°C water bath or at 99°C in a PCR machine, in order to open the secondary structures of the proteins. The samples were placed on ice to cool. To

separate out the different sized proteins a SDS polyacrylamide gel was run. For ease of use and consistency, pre-cast Nu-PAGE™ 4-12% Bis-Tris gels (Invitrogen™) were used. 1litre of 1x MOPS running buffer (20x purchased from Invitrogen™) was prepared. An XCell *Surelock*™ Mini-Cell electrophoresis unit (Invitrogen™) was set up and the protein samples loaded. 10µl of Rainbow recombinant molecular weight marker (Amersham Pharmacia Biosciences) were run alongside the protein samples and when possible, a positive control sample, to ensure the western blotting procedure had been successful. The gel was run at 200V for 50 minutes.

After the protein samples had run down the gel, the proteins had to be transferred to a nitrocellulose membrane in order to be probed with the appropriate antibody. A piece of Hybond-P™ polyvinylidene fluoride (PVDF) (Amersham Phramacia Biosciences) membrane was cut to the size of the gel as were 4 pieces of blotting paper. 1 litre of transfer buffer was prepared from a 20x stock with 10% (v/v) methanol (20x NuPAGE transfer buffer purchased from Invitrogen™). The PVDF membrane was pre-soaked in 100% methanol for 2 minutes and then washed in transfer buffer. The blotting paper and the sponge pads for the transfer unit were all thoroughly soaked in transfer buffer before assembly of the transfer cassette. The transfer was carried out using the transfer apparatus of the XCell *Surelock*™ Mini-Cell electrophoresis unit. The unit was set up as shown in the diagram below and inserted into the mini-cell unit so that the direction of the flow of the electric current was as shown below.

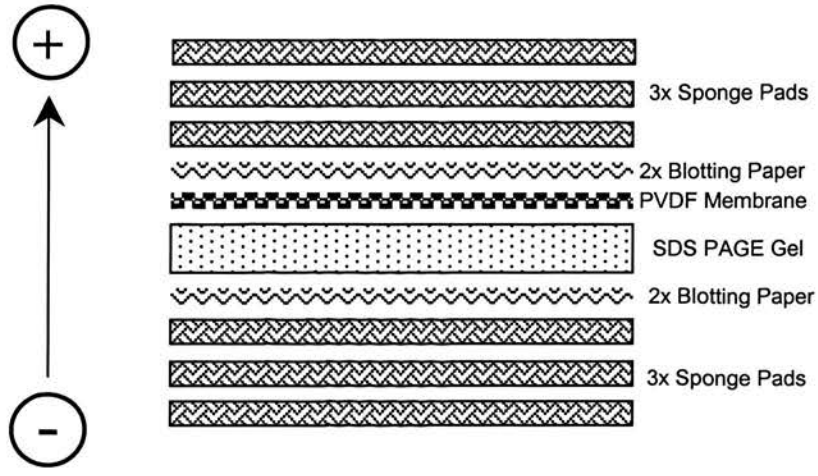

Figure 2.3 *Electrophoretic transfer setup of proteins in Western blotting.*

During the assembly of the unit, care was taken to eliminate any air bubbles between the layers that could interfere with the transfer. The tank was filled with the remaining transfer buffer; the transfer was carried out at 100 mAmps (25 volts approximately) for 1.5 hours. The membrane was checked for good transfer by the clarity of the rainbow marker transfer. The membrane was dried stored between blotting paper sheets at 4°C until the probing could be done.

2.3.3.2. Immunoblotting

The membrane was rehydrated in 100% methanol for 2 minutes and rinsed in dH_2O. To block non-specific antibody sites on the proteins on the membrane, it was incubated in a blocking solution of 5% (w/v) powdered, low-fat milk (Marvel), 1% BSA (v/v) in PBS. The membrane was left in this solution at 37°C for a minimum of 4 hours or overnight at 4°C with agitation. The membrane was washed in a solution of 1% (v/v) FCS and 0.1% (v/v) Tween20 in PBS for 10 minutes. This wash was repeated six times in fresh wash solution. This step was followed with two 5-minute washes in PBS alone. The primary antibody corresponding to the protein of interest was prepared to a pre-optimised concentration in freshly made blocking solution and applied to the membrane overnight at 4°C. The membrane was washed as before and a biotinylated secondary antibody was applied to the membrane at a 1/200 dilution in blocking solution. The membrane was left at room temperature for 2 hours with

agitation. Washes were done as before. Horseradish peroxidase (HRP) was conjugated to the biotinylated secondary antibody using the DAKO AB Complex/HRP system. The reagents were prepared according to the manufacturer's instructions for use in immunohistochemistry. The mixture was diluted 1/50 in PBS and the blot was bathed in the reagents for one hour at room temperature. Washes, as done between each incubation, were repeated. The blot was developed using the ECL Western Blotting detection kit (Amersham Pharmacia Biosciences). The manufacturer's instructions were followed in preparing the reagents and the blot was incubated in the mixture for one minute. The excess reagent was allowed to drip off the blot. The membrane was then wrapped in Saran wrap and exposed to Kodak X-OMAT™ LS film for several different exposure times varying from 10 seconds to 1 minute to get the best exposure and clarity as possible.

2.3.3.3. Stripping PVDF Membranes

In order to check that an equal quantity of protein was successfully loaded and probed in the Western blots, the PVDF membranes were stripped and reprobed for a ubiquitously expressed protein. Anti-mouse β-actin (raised in rabbit) (Santa Cruz Antibodies) was used in all cases with an HRP conjugated anti-rabbit secondary antibody (Sigma). The PVDF membranes were not allowed to dry out and if the reprobing was not immediately carried out, the membranes were stored in Saran wrap at 4°C. The membranes were immersed in 2% (w/v) SDS, 62.5 mM Tris, 100mM β-mercaptoethanol at 50°C for 30 minutes. The membranes were washed three times in PBS supplemented with 0.1% (v/v) Tween20 for 5 minutes each time. The membranes were blocked and reprobed following the same procedure as was applied to the initial probing.

Antibody	Isotype	Working dilution	Supplier
GFP, rabbit polyclonal	IgG	1:1000	Chemicon
β-actin, goat polyclonal	IgG	1:1000	Santa Cruz Biotechnology
Anti-rabbit HRP conjugated	IgG	1:500	Sigma
Rabbit anti-goat HRP conjugated	IgG	1:500	Sigma

Table 2.5 *Antibodies used in Western blotting.*

2.3.3.4. Quantification of Protein Concentration

To quantify the concentration of protein in relation to the total protein loaded, the Quantity One densitometry programme was used on the developed X-ray films of the blots using a BIO-RAD Fluor-S™ MultiImager.

2.4. Embryo Manipulation and Dissection

2.4.1. Blastocyst Injection

Jim McWhir carried out all blastocyst injections. Timed matings were set up between stud males and oestrus-selected female mice. Pregnant mice were killed by cervical dislocation and the uterine horns removed. 3.5-day-old blastocysts were flushed out of the uterine horns and collected by passing ES cell medium buffered with 20mM HEPES through the uterine horns with a syringe and a fine needle. Up to 15 ES cells were injected into the inner cell mass of the blastocysts which were transferred back to pseudopregnant mice to continue to term or until the developing embryos were collected to look at contribution of the ES cells.

2.4.2. Tetraploid Embryo Rescue

Tetraploid embryo complementation or tetraploid rescue involves the generation of a tetraploid embryo, incapable of contributing to the epiblast and the embryo proper. Tetraploid embryos are only able to contribute to the extraembryonic lineages. Following aggregation or blastocyst injection of ES cells into the tetraploid embryo, a tetraploid embryo-ES cell chimaera is generated with the ES cells obligated to form all the epiblast-derived cells, thus forming a completely ES cell-derived embryo. The cells from the tetraploid embryo are limited to the extraembryonic trophectoderm and visceral and parietal endoderms. By using genetically altered ES cells e.g. carrying a targeted locus, transgenic mice can be generated bypassing the requirement for several rounds of breeding litters (Nagy *et al.*, 1990; Nagy *et al.*, 1993). The viability of the ES-derived foetuses is dependent on the quality of the cell line. In most instances, the technique is used to look at a gestational phenotype and pregnancies are terminated prior to birth for examination. However, live births have been seen with particularly virile ES cell lines of low passage (Nagy *et al.*, 1993). This technique is especially useful in the generation and study of mice with multiple genetic mutations, which would take exponentially longer to generate via conventional breeding.

Jim McWhir carried out the procedures involved in tetraploid embryo rescue as well. Two cell embryos were released from the oviduct by tearing it apart. The embryos

were washed in M2 medium (Sigma) three times and placed in an M2 drop under mineral oil in a fusion chamber. The embryos were transferred to a drop of 0.3M mannitol supplemented with 50μM calcium chloride ($CaCl_2$) and 100μM $MgCl_2$ also in the fusion chamber. The embryos were placed between the electrodes, one at a time. They were orientated so that the blastomere contact wall was perpendicular to the electrodes.

The embryos were pulsed using the following settings: pulse amp 6.8, pulse length 0.8, AC amp 3, AC time 1 and number of pulses 1. The embryos were placed in M16 medium (Sigma) under oil and placed in a 37°C incubator. After 30-60 minutes had elapsed, the embryos were checked and any 2-cell embryos that had not fused to form 1-cell embros were removed. The fused embryos were left to develop in the incubator overnight.

The following day, 4-cell embryos were transferred through 3 washes with M2 medium (Sigma). The zona pellucida of the embryos was removed by washes in acidified Tyrode's solution until the zona pellucida had dissolved. The embryos were washed through 3 drops of M16 medium before being placed in individual drops of M16 medium (Sigma) with indents. The ES cells intended for tetraploid embryo rescue were lightly trypsinised so that they were disaggregated, but not quite at a single cell suspension. The ES cell clumps were washed through 3 drops of M16 before an ES cell clump of approximately 15 cells was placed beside an embryo in the indent of the M16 drop. A second embryo was placed on the other side of the ES cell clump to produce an aggregation sandwich. The aggregations were incubated overnight at 37°C. The following day, successfully rescued embryos should have compacted and should begin to form a blastocoel cavity. The blastocysts were transferred to uterine horns of pseudopregnant recipients. The embryos were harvested at a suitable time in development: typically 11 dpc.

2.4.3. Embryo Dissection

Mice were sacrificed as described above and the uterine horns cut out. For the purposes of this project, most dissections were carried out on post-implantation stage

embryos between days 7.5-12.5 dpc. The individual decidua were cut whilst still covered by the uterine horns. 10-12.5 dpc embryos could easily be isolated from the deciduum and yolk sac by a single incision laterally into the deciduum to pierce the yolk sac. Using forceps to enlarge this hole followed by the application of light pressure onto the deciduum, the embryo was usually forced out of the deciduum and yolk sac without any observed damage and with the torn yolk sac still attached. In 7.5-9.5 day old embryos, the yolk sac cavity was neither large enough, thin enough or fluid filled enough to attempt this. For these embryos, the deciduum was cut out of the uterine shell and prised apart very carefully with fine forceps.

2.4.4. Generation of Tumours in SCID Mice

Mice with severe combined immunodeficiency (SCID) (Harlan) were injected intramuscularly in the hindlimb with 10^7 ES cells in a maximum volume of 100µl of PBS, to test the cell population's pluripotency or to look at ES cell contribution and differentiation in tumour generation. Mice were sacrificed when a tumour was detected growing in the hindlimb and the tumour was dissected out. The tumour was sectioned and assessed for the presence of tissues from all three germ layers. If the pattern of ES cell contribution to cell lineages was being assessed, the tumour sections assessed for ES cell contribution, either by the detection of an ES cell specific marker e.g. GFP (green fluorescent protein) or LacZ.

CHAPTER 3

Generation of ES Cells Tagged at the *Flk-1* Locus for Selection and Isolation

Generation of ES Cells Tagged at the *Flk-1* Locus for Selection and Isolation

3.1. Introduction

The *flk-1* gene was first isolated in 1991 (Matthews *et al.*, 1991). A multipotential haematopoietic population from the foetal liver was enriched for by selection for AA4.1 and Sca-1 and against LIN antibodies. From this population, tyrosine kinase cDNAs were specifically amplified for the purpose of identifying molecules important for haematopoietic proliferation and differentiation.

The *flk-1* gene is expressed immediately after gastrulation leading to the direction of a subset of mesodermal cells to differentiate to form vascular tissues that are organised into the initially primitive blood vessels that serve the embryo. It is also expressed during neovascularisation in adults after severe wounding or during tumorigenesis. *Flk-1*'s importance was confirmed by gene targeting experiments, which revealed its expression pattern in ontogeny and its role in vasculogenesis (Shalaby *et al.*, 1995; Shalaby *et al.*, 1997). In homozygous mutants no blood islands were seen, no blood vessels formed and the haematopoietic progenitor population, although found not to be completely eliminated, was nonetheless almost undetectable and nonfunctional. Homozygous mutants died between 8.5 and 9.5 dpc and were resorbed by 10.5 dpc. It was clear that if vascular progenitors did not possess the FLK-1 receptor, they were unable to migrate to an appropriate site of differentiation where they would presumably receive further direction down the endothelial and haematopoietic lineages. However, beyond this activity, and that of other receptors such as *flt-1*, *tie-1* and *tie-2*, the molecular patterns of expression that drive the differentiation of mesoderm to the various cell types that eventually comprise the fully formed vasculature, are superficial and poorly understood. Transgenic approaches utilising *flk-1* to identify and track this population of cells could shed light on the mechanisms that are at play to direct cell fate beyond this stage of lineage specification and elucidate the signalling pathways at play.

Flk-1 is an ideal candidate to investigate the path of vascular development due to its association with the emergence of the putative haemangioblast. Its expression at the time of YS blood islands formation (7dpc), singles it out as a strong candidate to mark precursors of the haematopoietic and vascular lineages, and potentially the haemangioblast.

Flk-1 was chosen as the candidate marker of the haemangioblast. Transgenic approaches were employed to identify and isolate a *flk-1* expressing population of differentiated ES cells that would enable the tracking of differentiation of ES cells and potentially the haemangioblast.

3.2. Aims

To construct targeting vectors that allowed for the visualisation (using the viable GFP (green fluorescent protein) reporter) and purification (using the selectable HPRT gene) of *flk-1* expressing cells from a mixed population of differentiated ES cells.

To carry out homologous recombination in ES cells using the newly constructed vectors to derive ES cell lines that have been targeted at the *flk-1* locus.

To ensure that any targeted ES cell lines used to isolate pure *flk-1* expressing cell populations have maintained their pluripotency and karyotypic integrity after the targeting experiment has been carried out.

3.3. Experimental Plan

The *flk-1* targeting construct used to generate the *flk-1* null mutant ES cells and mice (**Figure 3.1**) was obtained from Dr Janet Rossant. It was modified so that a *flk-l* expressing cell population could be isolated using a promoter-trap strategy to drive either *gfp* or *hprt* gene expression using the *flk-1* promoter.

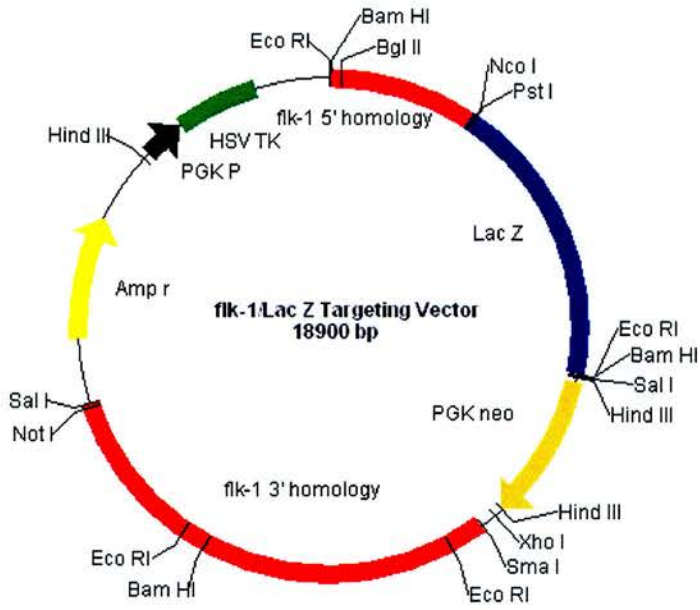

Figure 3.1 *The original targeting vector used to knock out flk-1 expression and track its expression pattern (Shalaby et al., 1995).*

3.3.1. Flk-1 Promoter Trap Strategy with an HPRT Reporter Gene

3.3.1.1. The Role of HPRT in Purine Biosynthesis

There are two biological pathways for purine biosynthesis. Purines can in most cases be generated via a system of recovering purines already in use in the cell via an existing salvage pathway of nucleotide conversion. However, there is also a less energy-efficient *de novo* synthesis pathway for purine synthesis.

Hprt is a housekeeping gene essential for the generation of purines via the salvage pathway. It catalyses the condensation of 5'-phosphoribosyl-1-pyrophosphate (PRPP) and the purine bases hypoxanthine and guanine to form 5'-inosine monophosphate (5'-IMP) and 5'guanosine monophosphate (5'-GMP) respectively (See **Figure 3.2**)(Krenitsky, 1969; Stout and Caskey, 1985).

The *hprt* gene is X-linked and therefore displays functional hemizygosity in male cells. This has simplified the generation of mutations that disrupt its function in male cell lines because loss of function is directly selectable. This has proved to be a

useful exercise in mammalian cell culture. The *hprt* gene has become a much-used tool in transgenic research as HPRT function can be either selected for or against. *Hprt* is able to bind to and ribosylate many purine analogues. This ability has been exploited to develop selection against the expression of HPRT using toxic purine analogues such as 6-thioguanine and 8-azaguanine (Stutts and Brockman, 1963). A selection regime for the expression of functional HPRT was also developed at around the same time. When exposed to a chemical cocktail of hypoxanthine, aminopterin and thymidine (HAT), any HPRT deficient cells should be killed (Szybalski and Szybalski, 1962). The hypoxanthine provides a purine source, the aminopterin acts to block the *de novo* purine synthesis pathway and the thymidine is supplied due to the additional inhibitory activity of aminopterin on the thymidine synthesis pathway, which has to be alleviated by the addition of the base. This results in the exclusive survival of cells capable of using the purine salvage pathway i.e. those with functional HPRT.

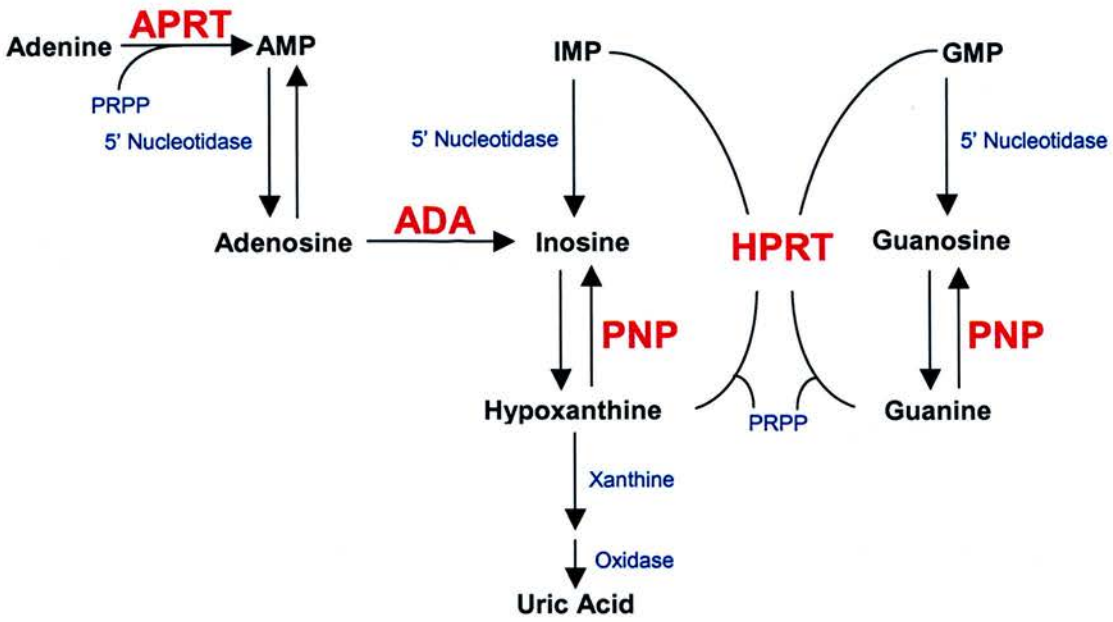

Figure 3.2 *The purine salvage and interconvension pathway. APRT, adenine phosphoribosyltransferase; ADA, adenosine deaminase; PNP, purine nucleoside phosphorylase; PRPP, 5'-phosphoribosyl-1-pyrophosphate. (Figure adapted from Stout and Caskey, 1985)*

By placing *hprt* under the transcriptional control of the *flk-1* promoter in the *hprt* deficient ES cell line: HM1 (Selfridge *et al.*, 1992), *flk-1* expressing cells could be isolated after differentiation by the application of HAT and the subsequent death of all non-expressing cells (**Figure 3.3**).

3.3.1.1.1. Advantages of using HPRT as a Reporter

HPRT is preferable to other chemical selection methods such as drug resistance because inhibition or selection for its activity has a notably faster mode of action as a result of the inhibition of a key pathway involved in cell survival. The neomycin phosphoribosyl transferase (*neo*) resistance cassette is probably the most predominantly used selection cassette but selection can take 7 days or longer to effectively kill all non-expressors of the resistance gene. Also, in recent years, several accounts of the destabilising effects of PGK*neo* on the transcription or regulatory control of adjacent genes to its site of integration have emerged that bring

into question the use of *neo* as a reporter in targeting experiments (Ramirez-Solis *et al.*, 1993; Rijli *et al.*, 1994; Fiering *et al.*, 1995; Olson *et al.*, 1996; Weissmann and Aguzzi, 1999).

3.3.1.1.2. Disadvantages of using HPRT as a Reporter

An issue that may present itself as a problem with this strategy is the short time period of *flk-1* expression and more importantly, the short time frame of the haemangioblast's presence. The length of time required for HAT selection to take effect will be crucial if the window for *flk-1* expression and haemangioblast isolation is to be met. In EBs, the haemangioblast is believed to exist between 2.5 and 3.5 days after formation (Kennedy *et al.*, 1997; Choi *et al.*, 1998), so if HAT selection takes several days to act, it may not be as effective a selective agent as desired. Nonetheless, HAT selection is still preferential to the use of antibiotic selection cassettes.

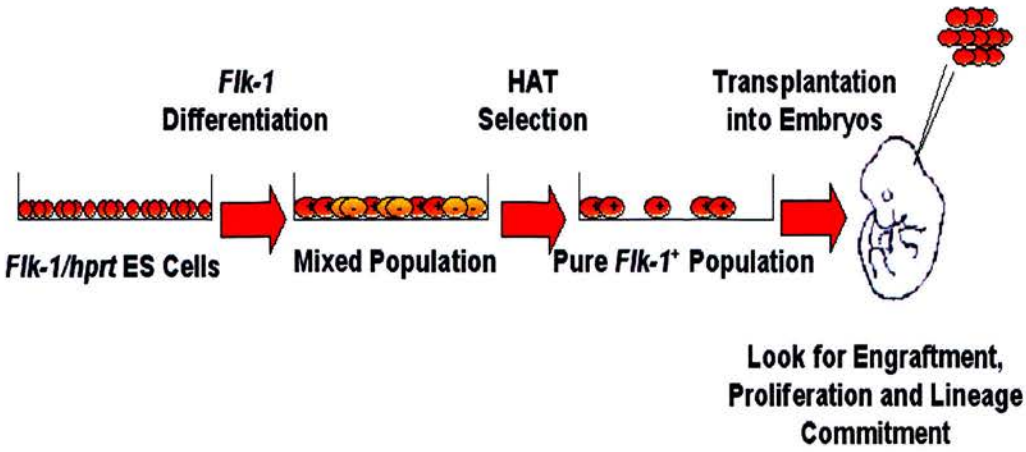

Figure 3.3 *Schematic diagram showing how selection for HPRT function in flk-1 targeted ES cells would enable the selection of a pure population of flk-1 expressing cells.*

Another issue to consider in the use of the *hprt* selection strategy is the possible bystander effect that could hamper the isolation of FLK-1 expressing cells. Metabolic exchange between neighbouring cells could result in the "kiss of life" phenomenon whereby phenotypically HPRT deficient cells are kept alive, despite

selection against them in HAT, due to the metabolic cooperation and exchange of purines from an adjacent HPRT expressing cell (Hooper and Subak-Sharpe, 1981). It may therefore be difficult to isolate a pure population if high cell density becomes an insurmountable problem. (If interaction, both via signalling or adherence to supporting cell lineages appears to be a key factor in the maintenance and self-renewal of a healthy FLK-1 expressing progenitor population, this bystander effect may not be a disadvantage).

3.3.1.2. Flk-1 Promoter Trap Strategy with a GFP Reporter Gene

In case the chemical selection for *flk-1* expression with an *hprt* reporter presented the problems mentioned above, a second targeting strategy was devised to place a promoterless EGFP (enhanced green fluorecent protein) reporter under the transcriptional control of *flk-1*. It was believed that this line could be a useful tool for visualisation of the progression of FLK-1 expression through differentiation, both *in vitro* and upon the generation of ES cell chimaeras *in vivo*. Also, using a GFP reporter allowed for the selection of GFP expressing cells using fluorescence activated cell sorting (FACS), thereby selecting for FLK-1^+ cells.

3.3.1.2.1. Advantages of GFP as a Reporter

The easy visualisation of the GFP protein *in situ* enables a simple method for identification of cells in which the protein has been transcribed and translated and thus indicates the activation of the gene of interest. The fact that no chemical interference is required for its detection means that GFP expressing cells can be observed for the length of time that the promoter of the gene of interest is actively transcribing the GFP reporter, without compromising the viability of the cells with stains or fixatives that are required for other commonly used reporters. GFP also has the advantage of being easily detected and selected for using FACS allowing for *in vitro* selection of a pure GFP expressing cell population.

3.3.1.2.2. Disadvantages of GFP as a Reporter

The half-life of the GFP protein is considered quite long (greater than 24 hours) which means that if a sensitive and precise indication of gene expression is required, it may not be ideal. However, several destabilised forms of the protein with considerably shorter half-lives have been developed to address this problem. For the purposes of this study half-life of the GFP protein was not a key issue as the focus was on isolating the earliest possible FLK-1^+ progenitor, not the dynamics of its expression. Although no publications have appeared to indicate that the phenomenon has been assessed quantitatively, anecdotal evidence (personal communications with several scientists) suggests that if the GFP transgene is expressed at high levels, it can have a cytotoxic effect on the expressing population. If true, this would establish a selective pressure in favour of low expressers or cells that have successfully silenced the transgene thus mitigating its advantages. Although easy visualisation of the protein's expression is a major advantage of the GFP reporter, autofluorescence is a common problem that arises that can either mask true GFP fluorescence or result in the questionable validity of results if GFP fluorescence cannot in some way be distinguished from autofluorescence. It is commonly observed in highly refractile cells, large and fluid-filled cells and in dense, three-dimensional structures or tissues.

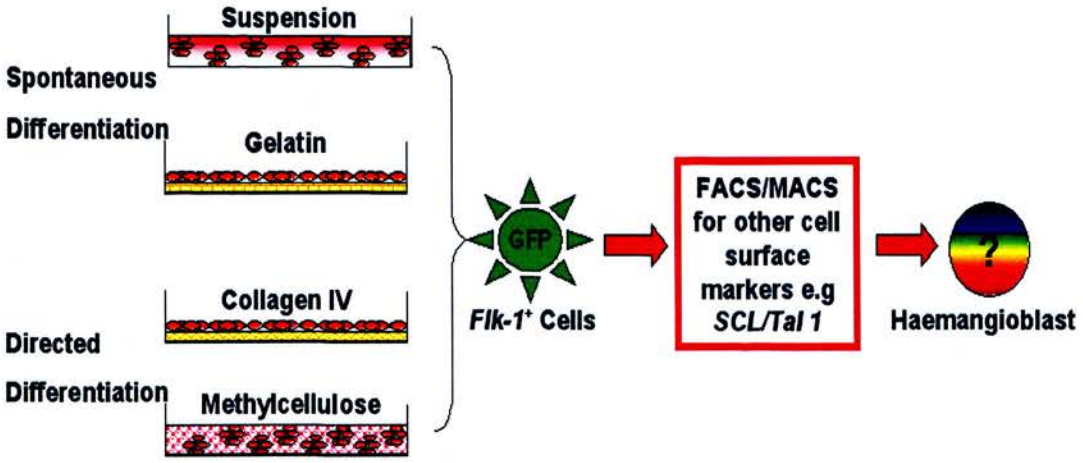

Figure 3.4 *Schematic representation showing how GFP under the transcriptional control of the flk-1 promoter can be used to compare spontaneous and directed differentiation protocols for induction of FLK-1, and how such cells could be purified and examined for other markers of the haemangioblast using FACS or magnetic assisted cell sorting (MACS).*

GFP expression can be both visualised and selected for using FACS (**Figure 3.4**). The GFP reporter was first proved in mammalian cells in 1994 (Chalfie *et al.*, 1994). It has since been used as a tool for the visualisation of expression patterns for many genes. It was therefore considered to be a good reporter gene to use for this project. Other groups have also used it as a tool to track haemangioblastic differentiation (Fehling *et al.*, 2003; Gilchrist *et al.*, 2003) since the inception of this project.

3.3.2. Targeting Vector Design

The initial targeting experiment carried out by Shalaby *et al.* (Shalaby *et al.*, 1995) replaced the translated part of the first coding exon of *flk-1*, together with the proximal portion of the first intron with a promoterless *lac Z* gene and a PGK*neo* (phosphoglycerate kinase promoter driving the neomycin phosphotransferase gene) selection cassette upon homologous recombination. Homologous recombination placed the *lac Z* gene under the transcriptional control of the *flk-1* promoter and disrupted *flk-1* expression (**Figure 3.5**). An external probe (**Figure 3.5**, pink box) was used to confirm targeting in conjunction with either a Hind III or Nco I digest.

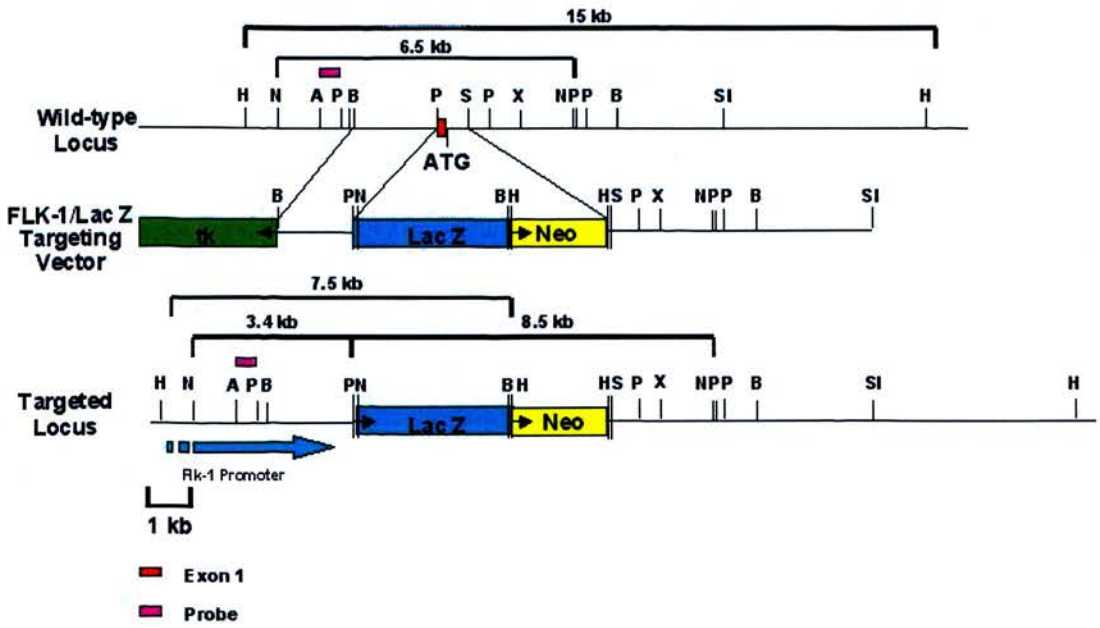

Figure 3.5 *Targeting strategy adopted by Shalaby et al. (1995) to determine the function of flk-1 during embryogenesis.* ***Lac Z****= β-galactosidase gene from Escherichia coli;* ***Neo****= neomycin phosphotransferase gene;* ***tk,*** *herpes simplex virus (HSV) thymidine kinase gene.* ***A****= Asc I,* ***B****= Bam HI,* ***H****= Hind III,* ***N****= Nco I,* ***P****= Pst I,* ***S****= Sma I,* ***SI****= Sal I,* ***X****= Xho I. Arrows specify the direction of promoter activity. Figure adapted from Shalaby et al. (1995).*

At the outset, the above targeting vector was to be modified by replacing the *lac Z* reporter gene with GFP or HPRT that was to fall under the control of the *flk-1* promoter upon targeting. However, the logistics of the cloning steps were problematic especially with regards to a necessary partial digestion step that was required to ablate a specific Hind III restriction site but which was never successfully carried out despite many attempts over several months. Ultimately, the necessary components from the above targeting vector were removed and the targeting construct was rebuilt in pBluescript SK+ (Stratagene®) (see **Appendix Ai** (HPRT) **and Aii** (GFP) for successful cloning strategies). The herpes simplex virus thymidine kinase gene used by Shalaby *et al.* for negative selection was omitted, as enrichment for targeted clones was deemed secondary to the speedy construction of the targeting vector, especially following accounts of disappointing levels of enrichment for targeting events. Initially, it was believed that enrichment factors were in the order of 1000x (Mansour *et al.*, 1988) however the advantages of positive/negative selection for enrichment have since been challenged with

enrichment being observed to be in the region of 2-20 fold (Mombaerts *et al.*, 1991). This has also been observed in the McWhir laboratory.

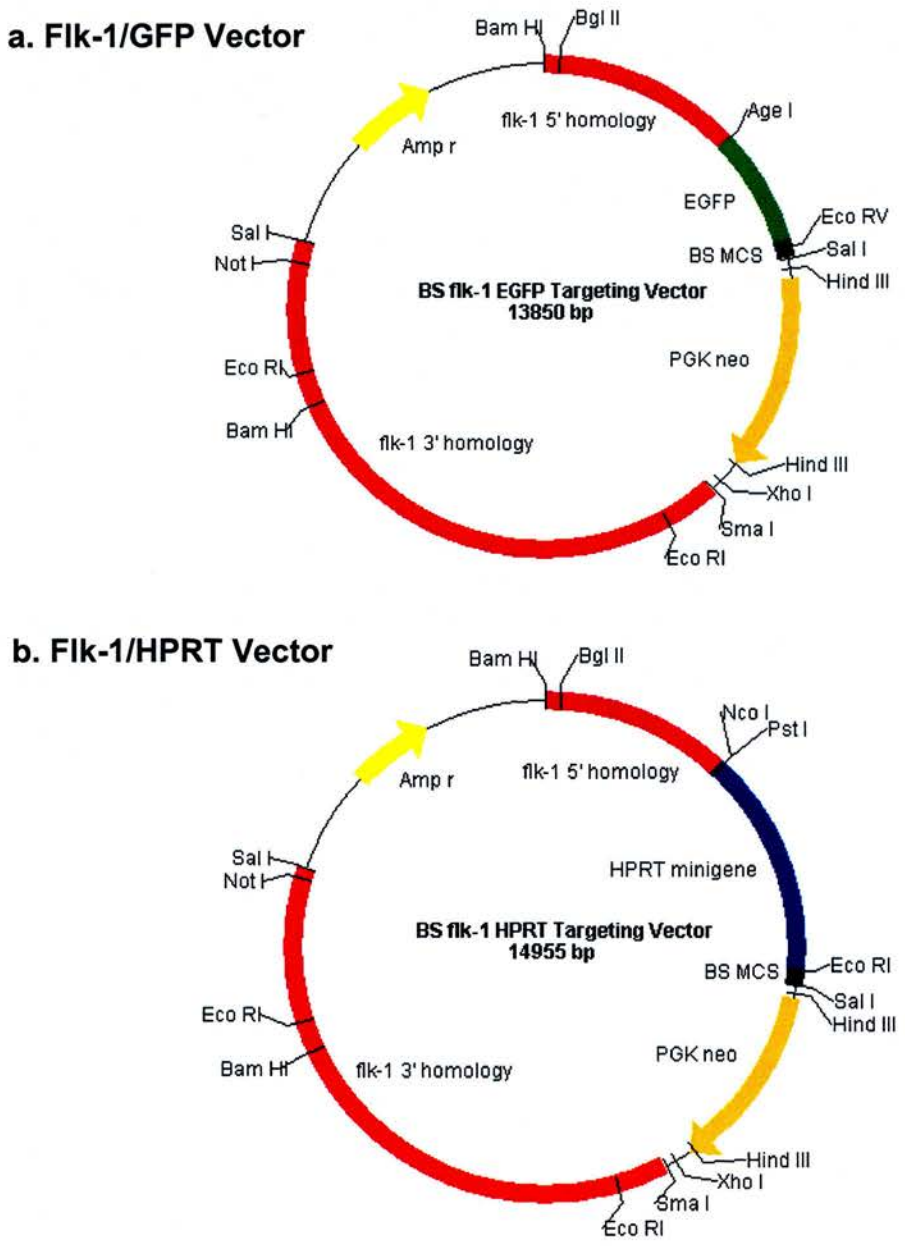

Figure 3.6 *Maps of the flk-1 targeting vectors: FLK-1/GFP and FLK-1/HPRT.* ***3.6a*** *the targeting construct designed to place the expression of EGFP under the control of the flk-1 promoter upon homologous recombination.* ***3.6b*** *the construct designed to enable the expression of the hprt minigene driven by the flk-1 promoter.*

On completion of both targeting vectors, the reporter genes were sequenced (DNASHEF) to ensure that no mutations had arisen during the subcloning steps. A single base pair mutation or loss could result in a premature stop codon leading to a truncated protein or a change in the open reading frame that would result in the incorrect synthesis of the GFP protein. Both reporters were found to have maintained their genomic integrity through the subcloning steps. The precise sequence for the *gfp* reporter gene is known, however, for the *hprt* minigene isolated from the DWM-1 plasmid (Melton *et al.*, 1986; Selfridge *et al.*, 1992) (See **Appendix Ai** for a plasmid map) the precise intron-exon boundaries are not known. The *hprt* minigene has been artificially constructed and only mRNA sequence, available in the sequence databases, was useful for mapping the minigene. The lack of information about the precise exon boundaries meant that the minigene sequences obtained after sequencing the targeting vector could not be matched with the mRNA sequence with complete confidence. There was a risk that a single base-pair loss or mutation at the end of an exon had occurred but was undetectable. Although the sequence information suggested the integrity of the minigene in the completed targeting vector, the gene's functionality could only be proved after a targeted ES cell clone had been isolated and tested for viability in HAT selection following induction of the *flk-1* gene.

Insertion of the reporter gene at the *flk-1* locus, causes the deletion of the ATG transcription initiation codon, the first exon and part of the first intron, leading to the ablation of *flk-1* expression at that allele, with the subsequent expression of the reporter gene on activation of the *flk-1* promoter. The remaining *flk-1* allele remains active. Both transgenes incorporated a polyadenylation signal 3' to the coding sequence for the reporters, ensuring appropriate transcription termination and preventing readthrough of the endogenous *flk-1* sequence.

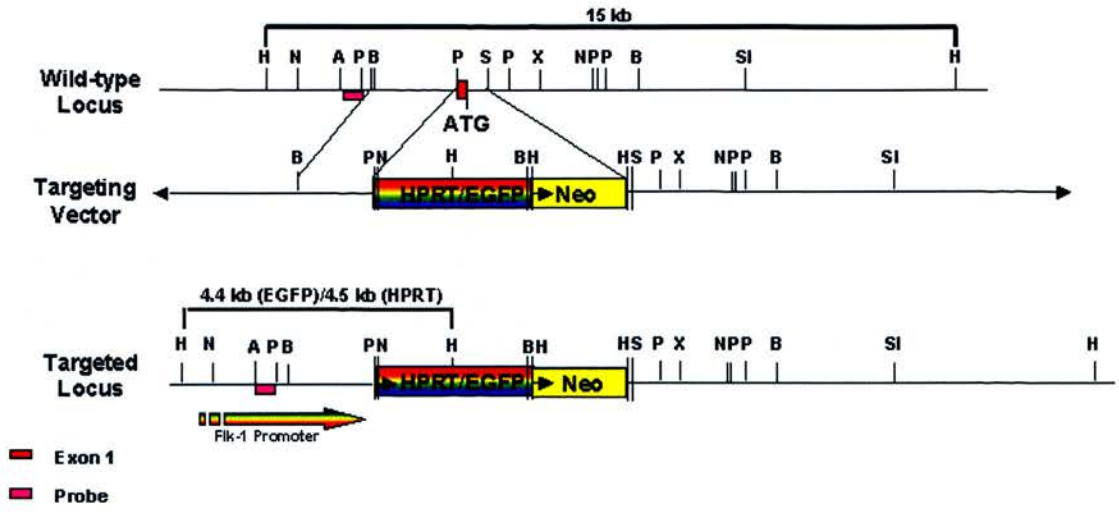

Figure 3.7 *Targeting strategies used with either HPRT and GFP vectors and the method of detection of homologous recombination used in both. Either a promoterless GFP or HPRT reporter cassette was inserted into exon1 of the flk-1 locus incurring a deletion of the first exon and the following intron. Recombinants were isolated by detection of the PGK-neo selection cassette with G418 and successful targeting was detected using a 5' external probe [pink box]. For both targeting strategies, following a Hind III restriction enzyme digest, wildtype cells produced a 15kb detectable fragment and targeted cells produced a fragment of 4.4kb and 4.5kb for the GFP and HPRT targeting strategies respectively. Figure adapted from Shalaby et al. (1995).*

The colonies were screened for targeting events by Southern blot following a Hind III restriction digest using the 5' external probe as shown in **Figure 3.7**. Attempts were made to devise a suitable 3' screening strategy to ensure single copy insertions and transgene integrity, however, much difficulty was encountered in the retrieval of the required sequence information. Despite the completion of the murine genome sequencing project (Waterston *et al.*, 2002), the genomic sequence for the *flk-1* gene is incomplete due to its location close to the centromere on the murine chromosome 5. Centromeric sequences are typically highly repetitive and difficult to sequence. Without resolution of the intron-exon boundaries 3' of the insertion site, such a strategy was not easily possible and therefore abandoned. Shalaby *et al.* also did not use a 3' probe in their confirmation of targeting.

3.4. Results

3.4.1. Initial Targeting Experiments at the *Flk-1* Gene Locus

All targeting experiments were carried out as described in the Materials and Methods. The targeting vectors were linearised by a Not I restriction enzyme digestion outside the arms of homology and insertion cassettes prior to the electroporation procedure. In **Table 3.1**, all targeting experiments carried out have been summarised along with their outcomes. The text that follows explains the rationale for each experiment and the significance of the result.

Cell Line Used for Targeting	Vector Used to Target Flk-1 Gene	Level of G418 Selection (µg/ml)	Col. Analysed (Col. Picked)	No. Targeted (% Targeted)
HM1	Lac Z (Original vector)	300	100 (100)	0
HM1	HPRT	300	497 (768)	2 (0.40)
		150	330 (576)	3 (0.91)
HM1	GFP	300	378 (480)	0
		150	316 (576)	3 (0.95)
R1	GFP	150	468 (576)	6 (1.28)
Ed1			75 (96)	0
Ed2			171 (192)	0

Table 3.1 *Tabulation of all targeting experiments carried out.*

3.4.1.1. Targeting Using the *Flk-1*/Lac Z Vector

While the *flk-1*/GFP and *flk-1*/HPRT vectors were being constructed, the targeting procedure was tested using the originally used (Shalaby *et al.*, 1995) *flk-1*/Lac Z targeting vector. A protocol for picking and analysing clones for targeting was used which involved growing each picked clone up to a 25cm^2 flask before freezing and

analysis (See Materials and Methods for details). 100 clones were picked, grown and analysed for targeting by Southern blotting, however no positives were found.

3.4.1.2. Targeting Using the Flk-*1*/GFP Vector

All targeting experiments from this point forth were carried out using a high throughput analysis method. This procedure enabled the screening of many more colonies, as the tissue culture maintenance of the picked clones was far less demanding.

A targeting experiment was carried out with the *flk-1*/GFP construct. 480 colonies were picked and screened for targeting as described in the Materials and Methods chapter. The G418 selection used was 300μg/ml. This was the standard concentration used in the McWhir laboratory for selection of possible homologous recombination candidates in murine ES cells. Out of these 480 clones, 378 survived the picking and lysis procedures and generated a discernable result in a Southern screen. The majority of the colonies grew well and survived trypsinisation for freezing of cell stocks. The procedure for carrying out an ethanol precipitation of genomic DNA was difficult in the 96-well plate format and resulted in the loss of several precipitated DNA pellets at the 70% ethanol wash stage. Ultimately, as with the targeting experiment with the original *lac Z* targeting construct, no positive clones were identified.

3.4.1.3. Targeting Using the Flk-*1*/HPRT Vector

A targeting experiment was carried out using the HPRT targeting construct. 768 colonies were picked into eight 96-well plates and G418 selection was applied at 300μg/ml. 497 generated a result by Southern screen: 64.7% of the picked colonies. Out of these, there were two targeted clones. The relative targeting efficiency (measured as number of targeted cells out of all colonies picked) was therefore 0.4%.

Although targeted clones were obtained from this experiment, it was nonetheless at a far lower level than expected. Shalaby *et al.* had reported a targeting frequency of 1

in 7 (14.3%) using Southern blot screening in 1995 using a targeting construct from which the exact same regions of homology and selection cassette were utilised.

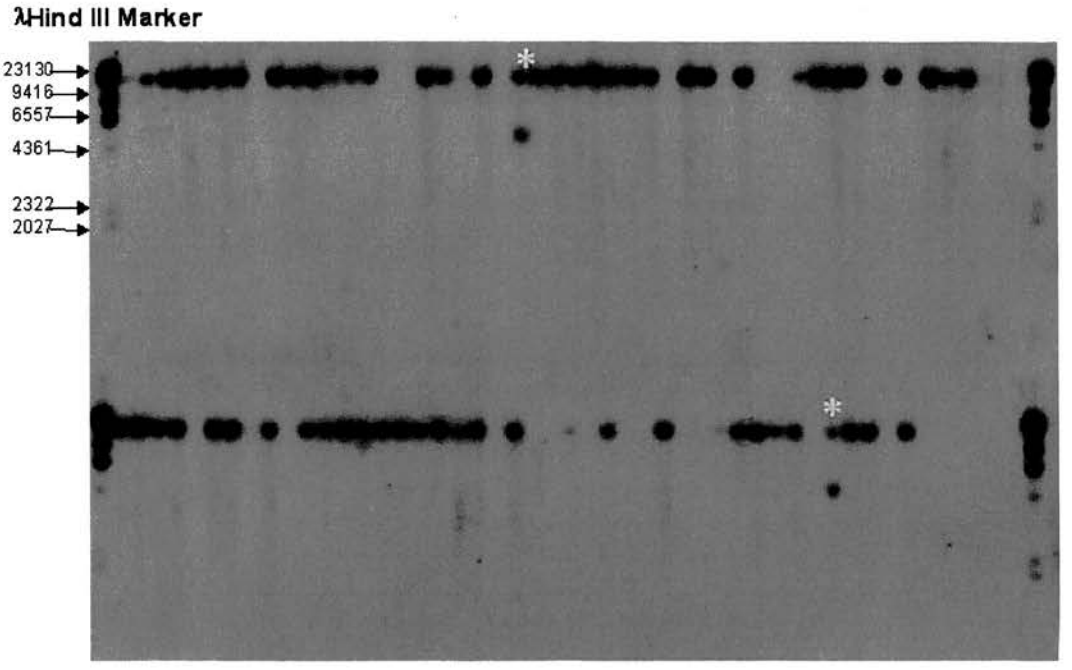

Figure 3.8 *A typical Southern blot screen of picked colonies from a targeting experiment at the flk-1 locus using the flk-1/GFP vector. Following a Hind III restriction enzyme digestion, wildtype colonies are detectable with a 15kb fragment. Targeted colonies [2, yellow asterisks] are detectable with a 4.4kb probed fragment.* ***Figure 3.4*** *shows a schematic of the targeting strategy. The λHindIII marker was run at both ends of the gel and also probed. Empty lanes are due to the loss of colonies or the DNA pellet prior to analysis.*

To check whether the resistant colonies that had been picked were "true" neomycin resistant colonies and not artefacts of a poor selection regime, two blots used to check for targeting were reprobed for the presence of the PGK*neo* cassette. Over 90% (130/140 colonies were positive for PGK*neo*) of the colonies picked showed the presence of the cassette (data not shown). It was hypothesised that the low targeting efficiency arose because the high level of G418 used was selecting against targeted clones and in favour of the random integration of the PGK*neo* selection cassette. This is especially likely if the *flk-1* locus has a downregulating influence on *neo* expression levels.

The original Shalaby targeting papers did not indicate the level of selection that was used to isolate candidate targeted clones. Dr Masatsugu Ema in Dr Janet Rossant's

laboratory was able to confirm that a G418 concentration of 150μg/ml had been used to isolate candidate colonies and that they carried out the majority of their targeting experiments with this level of G418 selection.

3.4.2. The Effect of Reducing Selection Levels on Targeting Efficiency

The targeting experiment using the *flk-1*/GFP vector was repeated as no targeted clones had been generated. At the same time, another targeting experiment was carried out with the *flk-1*/HPRT ES cell line despite the isolation of two targeted clones from the first round of targeting experiments. By repeating targeting experiments using both targeting vectors in parallel and a common source of ES cells could help to ascertain whether the low frequency of homologous recombination observed in the previous experiments were isolated incidents attributable to the state of the cells used, or establish if there was a difference between the efficiency of the targeting vectors which would implicate the reporters in determining the rate of homologous recombination; both targeting experiments were therefore repeated. The only variable that was altered between the previous experiments and these was the level of selection that was applied to the cells which was reduced from 300μg/ml to 150μg/ml. For each of the targeting experiments 576 colonies were picked. These were grown up for cryopreservation and the extraction of total genomic DNA as before and analysed for homologous recombination at the *flk-1* locus using the same Southern screen. In the targeting experiment using the HPRT targeting construct, a total of 330 colonies were successfully analysed with 3 positive colonies being isolated. In the experiment using the GFP vector, 316 colonies were successfully analysed with 3 positive colonies also being derived from this experiment (**Table 3.1**). **Figure 3.8** shows a typical Southern blot with two colonies that were found to be targeted.

3.4.3. The influence of DNA Isogenicity on Targeting Frequency

Shalaby *et al.* (1995) reported a targeting frequency at the *flk-1* locus of 1 in 7 of all colonies analysed by a Southern screen: a percentage targeting frequency of 14.3%.

This is approximately 35 times greater than the frequency observed in these targeting experiments carried out in HM1 ES cells.

The targeting vectors used in these experiments had exactly the same homology taken from Shalaby's targeting vector that induced a very high level of homologous recombination; it was unusual that there was such disparity between the efficiency of homologous recombination. Shalaby *et al.* did use negative selection with HSV-TK however they did not clarify how many colonies they obtained before the application of gancyclovir or what level of enrichment they had or expected to have. They would have to have gained a selective advantage of over 30 fold to explain the paucity of targeting in this experiment.

The most plausible reason for the low efficiency of homologous recombination was the use of a non-isogenic ES cell line for the targeting experiment. The *flk-1* arms of homology originally used were drawn out of a 129Sv cDNA library (Shalaby *et al.*, 1995) and used to construct the targeting vector. Their targeting experiment was carried out using R1 ES cells, which were derived from 129/Sv mice and were thus syngeneic with the arms of homology used. The ES cell line used in this experiment was the HM1 ES cell line. This was a rederivation of the E14TG2A ES cell line, which in turn was derived from 129/Ola P2 mice (Selfridge *et al.*, 1992). It appeared unlikely that the DNA would not be syngeneic, firstly, because 129/Sv and 129/Ola are substrains of the 129 strain of mice and therefore less likely to possess polymorphisms between each other than other inbred mouse strains; secondly, as *flk-1* is essential for the development of the vasculature and is expressed throughout ontogeny, it was likely to be highly conserved between species. However, noncoding intronic regions, absent of regulatory elements could still be subject to high mutation levels that would be uncontrolled due to their location but which could still have a profound impact on the level of homology in the arms of homology of the targeting vectors between intraspecies strains of mice (Kostyniak, 1980; Dizik *et al.*, 1982).

The requirement for syngeneic DNA for the best homologous recombination frequencies has been discussed (Hooper, 1991). To evaluate this possible explanation of the low targeting frequency and to obtain additional targeted clones, three more targeting experiments were carried one. Using only the *flk-1*/GFP targeting vector, R1 ES cells (used by Shalaby) were electroporated exactly as before. Also two F1 lines: Ed1 and Ed2 isolated by Dr Ed Gallagher from blastocysts obtained from matings of C57Bl/6 and 129/Sv mice were electroporated. Ideally a completely outbred ES cell line would have been best for carrying out this experiment, or at least an F1 population from a cross that did not include a 129 mouse strain. However there was no access to such an ES cell line. Few non-129 strain derived ES cell lines exist in the murine system due to possible epigenetic differences or other as yet unknown characteristics of other mouse strains that make them less amenable to ES cell isolation. If genetic syngeneity had played a part in the low level of homologous recombination, the number of targeted clones obtained from the R1 ES cells would have been expected to be far greater than the number of targeted clones obtained from HM1 ES cells, which in turn would be expected to have had more successful homologous recombination than the outbred lines (although the fact that the cross included 129/Sv may have meant that the *flk-1* locus had remained isogenic with the 129/Sv arms of homology).

Table 3.1 shows that the R1 ES cell line which was syngeneic with the DNA sequences in the targeting vector homologies yielded 6 targeted clones with a targeting frequency of 1.28%. In HM1 ES cells, the targeting frequency was 0.95 %. For both these ES cell lines, many more colonies than were picked, appeared after selection. This contrasted with the poor colony yield for the F1 ES cell lines Ed1 and Ed2, which yielded 96 and 192 colonies respectively, all of which were picked and analysed. No targeted colonies were isolated from these lines. The results of this experiment comparing targeting frequencies in different ES cell lines did not clarify the low targeting frequency observed in previous experiments. Although it did not disprove the hypothesis that syngeneic DNA aids the efficiency of homologous recombination, the number of clones screened was insufficient to yield enough targeted clones to allow the results to be interpreted with statistical significance.

3.5. Discussion

3.5.1. Possible Reasons for Low Frequency of Homologous Recombination

A high targeting frequency of 14.3% had been observed when the flk-1 locus was previously targeted with a vector sharing the same homology regions and selection cassette as the vectors used in these experiments (Shalaby *et al.*, 1995). The best targeting frequency obtained using the modified *flk-1* targeting vectors was 0.95% despite the screening of over 500 colonies for each of the two developed targeting vectors.

There were several possible reasons for the extremely low targeting efficiency. Once the vectors had been constructed, the *gfp* and *hprt* reporter genes that would fall under the control of the endogenous *flk-1* promoter were sequenced to ensure that the sub-cloning steps had not incurred mutations in the genes that could affect the expression of the reporters. The PGK*neo* had not been sequenced, as the ability to isolate a number of colonies after selection was applied in a targeting experiment was a sufficiently good indicator of its integrity. The arms of homology were also not sequenced. If mutations had arisen in the arms of homology during the subcloning steps, they could have impacted on the frequency of homologous recombination. However, the process of finding the homologous sequence is not rate limiting as shown by Thomas *et al.*, 1986 who showed that increasing the concentration of the targeting vector or the number of target sequence copies did not increase the efficiency of targeting. In retrospect, it is clear that the homologies should have been sequenced to ensure that they had maintained their integrity through the cloning steps in vector construction.

Despite the success of Shalaby and colleagues to target the *flk-1* locus, cell line-specific variations could have led to the poor targeting frequencies achieved in these experiments. It was assumed that *flk-1* was not expressed in ES cells therefore it was conceivable that the region in which the *flk-1* gene was situated was therefore heterochromatinised. This may have left the *flk-1* locus impenetrable by recombinases and could have meant that homologous recombination was less likely

to occur. However, experiments that will be discussed in Chapter 4 have since elucided that the level of *flk-1* expression in undifferentiated ES cells is quite dynamic and varies noticeably without apparent differentiation or other identifiable reason.

An additional possibility, referred to earlier, is that the PGKneo selection cassette induced the heterochromatinisation of that region, including the cassette itself, thus preventing the isolation of true targeted clones (Ramirez-Solis *et al.*, 1993; Rijli *et al.*, 1994; Fiering *et al.*, 1995; Olson *et al.*, 1996; Weissmann and Aguzzi, 1999). Random integration could still occur freely as it employs a different mechanism to take place and integration in such instances would take place preferentially at open sites that are favourable for recombination.

As already mentioned, it is known that the processes of random integration and homologous recombination are favoured at two distinct phases of the cell cycle and act independently of each other. Homologous recombination efficiency is believed to be optimal at early to mid S-phase in the cell cycle (Wong and Capecchi, 1987) whereas random integration is thought to peak at G2/M phase (Yorifuji *et al.*, 1989). Cotransfection experiments have shown that random integration and homologous recombination rarely occur in the same cell (Reid *et al.*, 1991). The pace of the cell cycle may therefore have a significant influence on the rate of homologous recombination independently of other possible influences. It is therefore possible that if the cells used in the targeting experiments had been electroporated at the point of the cell cycle when random integration was favoured, the frequency of homologous recombination would be very low.

3.5.2. The Significance of Maintaining Genomic Isogenicity in the Execution of Targeting Experiments

It is known that sequence mismatches between the arms of homology of a targeting vector and the endogenous site for homologous recombination reduce the targeting efficiency, especially if the region of mismatch is large (Waldman and Liskay, 1988).

It has also been observed that if the arms of homology between a targeting vector and the genomic locus are not isogenic, the rate of targeting is affected. Homologous recombination at the retinoblastoma susceptibility locus in E14 ES cells was 20x more efficient with a targeting construct derived from the same strain 129 than with one derived from a different strain BALB/c (te Riele *et al.*, 1992). It is therefore preferred if targeting vectors are designed using homologies derived from the same strain of mouse.

As most ES cell lines are derived from the 129 strain of mouse, and in this particular instance the arms of homology and the ES cell line were both from a 129-strain mouse, this was not considered to be a problem. However, in light of the low efficiency of homologous recombination, the possibility of there being polymorphisms at the *flk-1* locus between the 129/Sv and 129/Ola substrains of mouse was considered.

Udy *et al.*, 1997 reported that they found that there was an inverse relationship between targeting frequency and cell doubling time with the R1 ES cell line showing the highest level of homologous recombination with a targeting frequency of 1 in 50. Contrary to other reports (Deng and Capecchi, 1992; van Deursen and Wieringa, 1992; te Riele *et al.*, 1992) where isogenicity was reported to be able to increase the frequency of homologous recombination from 4 to 25 times its rate otherwise, the authors did not find that isogenicity of DNA between cell lines and vector DNA impacted on the targeting frequency. They accounted for the observations others made with respect to isogenicity by considering the cycling capabilities of various cell lines and how this would affect the rate of homologous recombination.

The targeting experiments in the R1 (129/Sv) and F1 (129/Sv x C57Bl/6) lines did not clarify the importance of isogenicity. It was clear that the R1 ES cells were more amenable to recombination with this vector as more colonies were generated after selection for recombination events. Both Ed1 and 2 produced lower numbers of colonies showing a lower level of recombination. However, this can be attributed to their slower cycling capabilities as both lines grew much slower than HM1s or R1

ES cells. The total number of colonies screened for the R1 ES cells was comparable with the number screened in the HM1 ES cells. As these results were not corroborated with additional repeats, no statistically significant conclusions can be inferred from the data. It is interesting to consider the possibility that the cycling of the ES cells determines the efficiency of homologous recombination however as this could account for the low frequency seen in HM1s. Although this ES cell line is comparatively stable and proliferates well, the R1 ES cell line, known to be less stable karyotypically (Gao *et al.*, 2002), proliferates noticeably faster so that at any time that the cells are harvested for electroporation, you are likely to get some cells in S-phase when homologous recombination is optimal. With a slower cycling population, timing would be more critical as cell activity would be slower with fewer cells in S-phase at any given time.

3.6. SUMMARY

- A previously used targeting vector used to target the *flk-1* locus using a promoter trap strategy to express Lac Z was modified so that upon activation of flk-1 expression, either the *gfp* or *hprt* genes are transcribed.

- Targeting experiments in mES cells enabled the isolation of targeted ES cell lines although the targeting frequency was surprisingly low.

- Investigations were carried out to establish whether the non-isogenic arms of homology of the targeting vectors were responsible for the low targeting frequency. These were inconclusive, as the observed rates of homologous recombination were not confirmed by repetition of the experiments, which, if carried out, would have allowed the statistical analysis of the data.

CHAPTER 4

Analysis of *Flk-1* Targeted Cell Lines

Analysis of Flk-1 Targeted Cell Lines

4.1. Introduction

Different ES cell lines frequently display variations in cellular morphology, growth behaviour and differentiation capacity and patterns. This is affected by the process of ES cell isolation and the stresses associated with that together with potential variation across the ICM at the time of ES cell isolation that could contribute to incidental differences between lines. Targeted clonal ES cell lines display a similar behavioural difference after being single-cell cloned. It is possible that individual cells within a stem cell population accumulate phenotypic variations over time. These could be associated with sporadic mutations incurred due to a gradual increase in passage number and possibly a decrease in telomere length, or there could be mutations favoured and therefore maintained in the cell population such as increased proliferative capabilities due to loss of cell cycle checkpoints. The process of targeting may also have induced a small chromosomal change e.g. a deletion or gene conversion event that affects the behaviour of a single cell that is clonally isolated. A mutant or aneuploid cell that has increased proliferative capabilities due to cell cycle loss of checkpoints e.g. transformed cells can overtake an entire culture within the course of a few passages. Before using any cell lines, it is therefore necessary to check that they are karyotypically normal. Multiple random insertions of the reporter that disrupt unknown genes are also a possibility that has to be considered as a cause for clonal variation between targeted cell lines, however in the absence of a 3' screen to check for this, there is no way to know if this has occurred. Multiple insertions are commonly associated with transgene silencing.

4.1.1. Karyotype Analysis of the Targeted Cell Lines

Out of the total of 8 targeted clones identified by Southern blot analysis, only 6 were successfully thawed from the 96-well plates. One clone for each of the reporters was lost; the *flk-1*/HPRT one due to complete differentiation after being thawed and the *flk-1*/GFP line due to the loss of the line whilst being resuscitated.

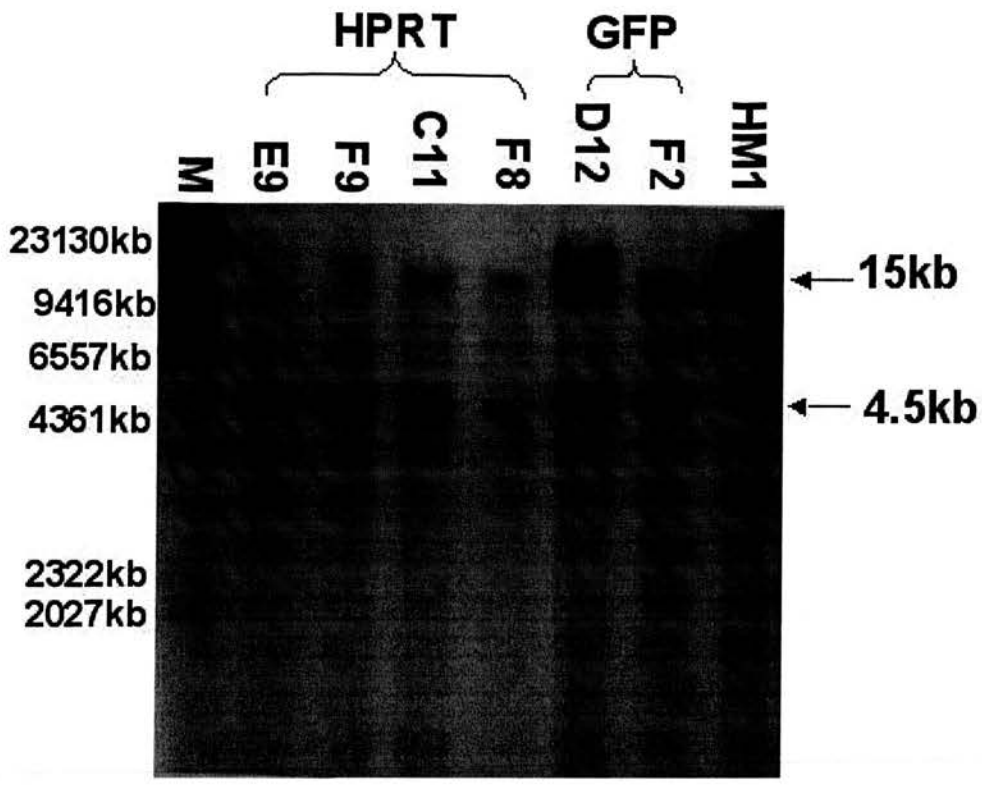

Figure 4.1 *Southern blot confirming the targeting events in 4 flk-1/HPRT and 2 flk-1/GFP cell lines. Figure 3.4 describes the targeting and verification strategy used to identify targeting events. Wildtype DNA corresponds to a 15kb probed band. Targeted cells produce one 15kb band corresponding to the remaining wildtype allele and one 4.4kb (GFP) or 4.5kb (HPRT) band corresponding to the targeted allele.* ***M*** *= λ/HindIII size marker.* ***HM1*** *is the parental wildtype cell line.*

For each remaining targeted clone, 50 metaphase spreads were analysed for the expected 40 chromosomes (**Figure 4.2**). Out of the 6 targeted clones only the *flk-1*/GFP line, D12 and the *flk-1*/HPRT line E9 were observed to have any aneuploid chromosomal counts (**Table 4.1**). A low frequency of observed abnormalities such as these, can be attributed to the chance loss of a chromosome in the preparation of the slides. If a true chromosomal loss had taken place, it would be expected that the abnormality would be far more prevalent in the cellular preparations due to the fact that such aberrations commonly result in a loss of cell cycle control and thus proliferative control leading to abnormal cells taking over a population of normal cells.

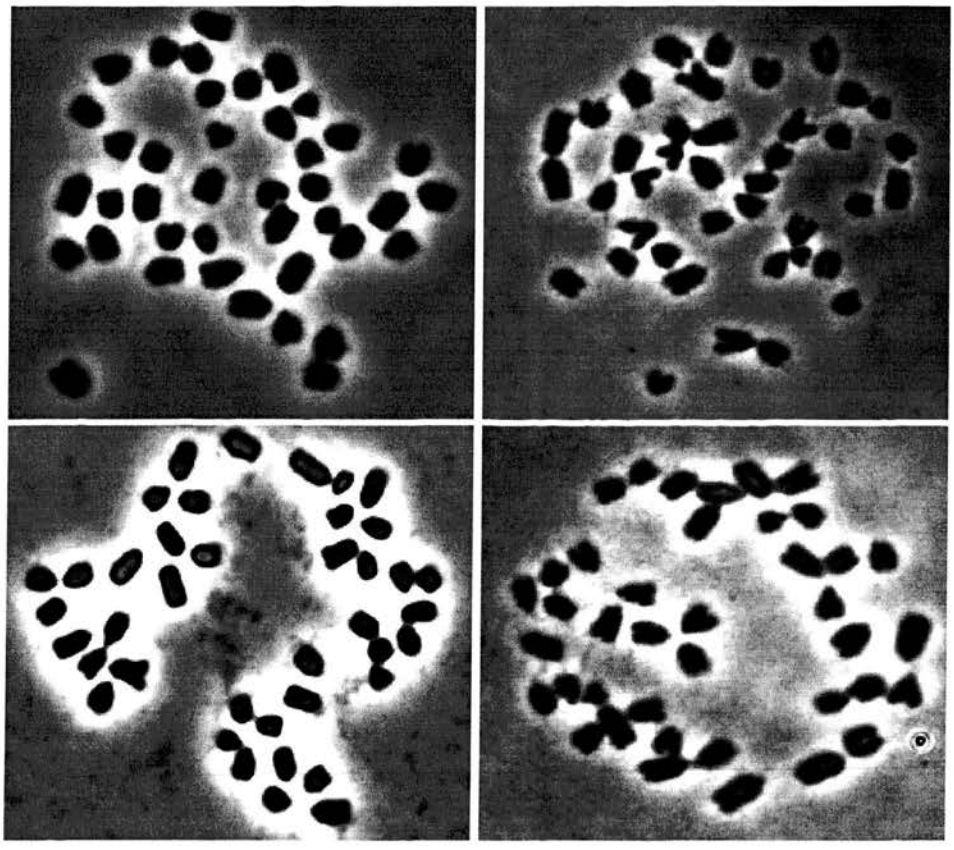

Figure 4.2 *Typical metaphase spreads counted to ascertain the chromosome number of the isolated targeted clones.*

Targeted Clone	Reporter	Number of Euploid Metaphase Counts/50	Number of Aneuploid Metaphase Counts (Evans, 1972)d)
D12	GFP	48	1 (39), 1 (41)
F2	GFP	50	0
C11	HPRT	50	0
F8	HPRT	50	0
E9	HPRT	49	1 (39)
F9	HPRT	50	0

Table 4.1 *Karyotypic analysis of all targeted clones*

4.1.2. Vigour and Self-Renewal Capabilities of Targeted Lines

Despite being derived from the same HM1 ES cell line, the various targeted clones displayed noticeable differences in recovery after trypsinisation or growth at low density. Three lines grew slowly in comparison to the others: the *flk-1*/GFP line F2, and the *flk-1*/*HPRT* lines C11 and F9. All three of these lines, as well as growing slowly, had poor plating efficiencies after trypsinisation and readily differentiated if they were seeded at and grown at low densities.

Initial experiments using all the targeted clones indicated that some lines were more consistent than others with respect to speed and course of differentiation. The two lines that were both vigorous and the most consistent were D12 for the *flk-1*/GFP line and F8 for the *flk-1*/*HPRT* line. It was decided to focus on these lines in the more complex experiments carried out so that observations could be accepted and interpreted with the greatest level of confidence possible.

4.1.3. Pluripotentiality of the Targeted Cell Lines

In order to ensure that the ES clones had retained their pluripotency following homologous recombination, the most robust of the isolated clones for each of the targeting experiments: D12 for the *flk-1*/GFP lines and F8 for the *flk-1*/*HPRT* lines, were used to generate tumours in SCID mice as outlined in Chapter 2.

D12 tumours were generated in 4 mice in 2 separate trials. In all instances, they consistently produced fluid-filled cystic tumours with poor cohesive structure. It is not clear why this was the case or whether it could have been due to the stress of targeting or an aberration brought on by the process. F8 tumours were generated in 2 animals in a single trial and were more typical of other ES lines producing solid tumours that could be easily dissected and sectioned with a variety of tissue types forming.

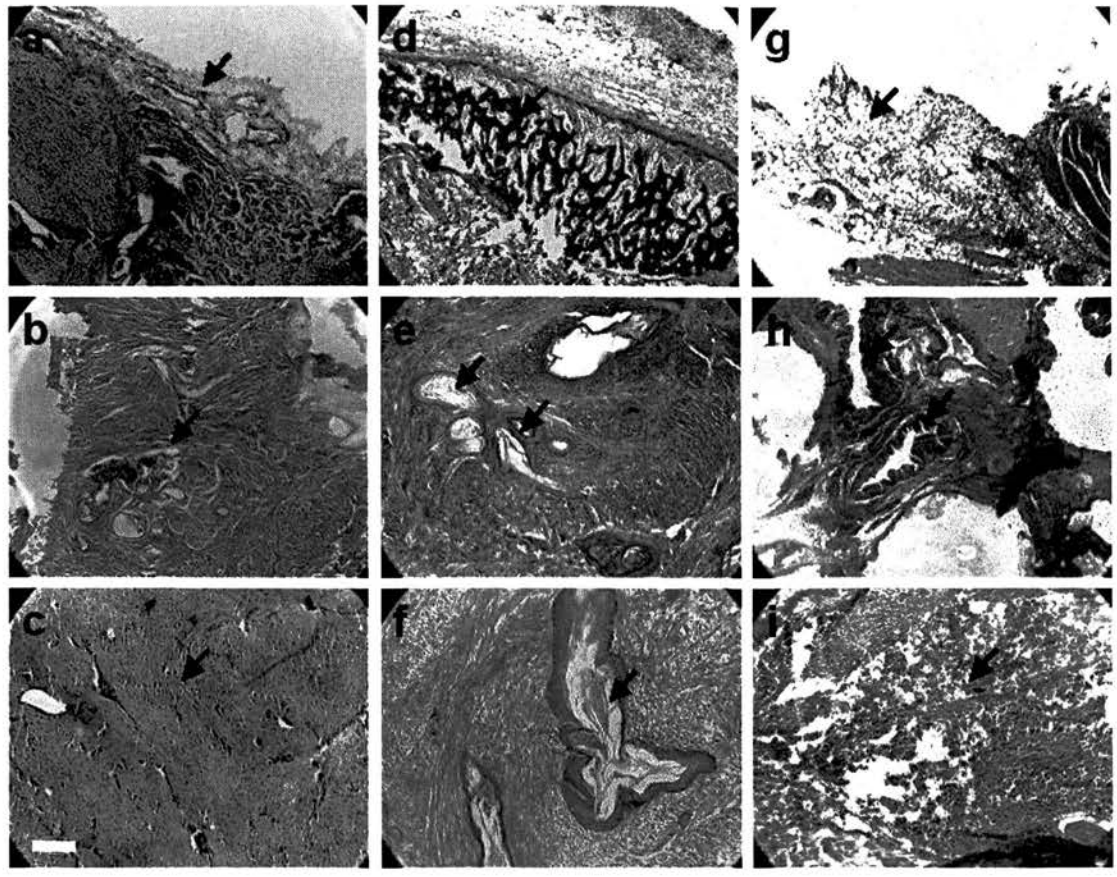

Figure 4.3 *Haematoxylin and eosin stained sections obtained from tumours generated in SCID mice from the injection of the targeted ES cell lines F8 (flk-1/HPRT).* ***a*** *epithelium,* ***b*** *muscle and collagen fibres,* ***c*** *cartilage,* ***d*** *gut epithelium typical of jejunum,* ***e*** *secretory ducts,* ***f*** *vein surrounded by muscle and connective tissue ,* ***g*** *bone,* ***h*** *gut mucosa typical of ileum,* ***i*** *cells of the brain stem . Scale bar represents 100μm.*

In both the tested ES cell lines, cells from each of the 3 germ layers was observed (**Figure 4.3**). Mesoderm was frequently observed with muscle and collagen fibres (**b**), cartilage (**c**), and blood vessels (**f**). Endodermal lineages including epithelia (**a, d**) and secretory glands (**e**) were also seen. Least frequently observed were ectodermal cells although cells from the brain were identified (**i**).

4.1.4. Generation of Chimaeras from D12 *flk-1/GFP* ES Cells

Both the D12 and F2 *flk-1*/GFP targeted ES cell lines were used in blastocyst injection to generate chimaeras that could transmit the *flk-1* targeted allele through the germline to generate heterozygous mice for the *flk-1*/GFP allele.

Male chimaeras were successfully identified from coat colour chimaerism. The embryos used were C57Bl/6 embryos, which give rise to animals with a dark coat colour; the HM1 ES cell line is derived from 129/Ola P2 mice that have a paler chinchilla coat colour. **Figure 4.4** shows coat colour chimaerism following blastocyst injection with the targeted ES cells. One chimaera was a 100% transmitter, generating a completely chinchilla coloured mouse (no photograph available).

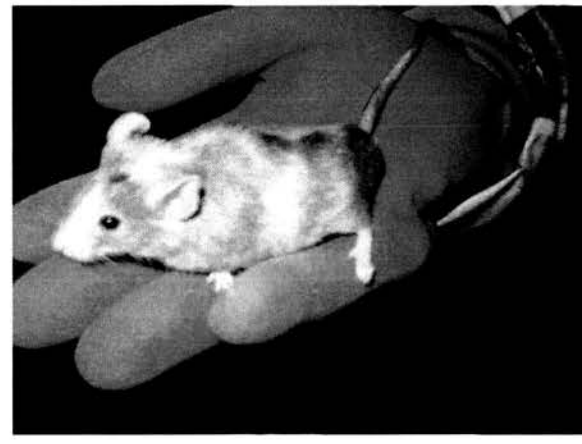

Figure 4.4 *Coat colour chimaerism after blastocyst injection of CBA mice (coat colour: agouti) with flk-1/GFP ES cells is derived from HM1 ES cells from 129/Ola P2 mice (coat colour: chinchilla).*

4.2. Faithful Expression of Transgenes upon *Flk-1* Promoter Activation

4.2.1. Introduction

To check that the *gfp* transgene was being correctly expressed and regulated, *flk-1* expression was stimulated *in vitro* and observed to see whether the transgene was

being expressed appropriately. HPRT-tagged cells could not be visualised *in vivo*, however, differentiation of the HPRT-tagged cells down haemangioblastic lineages would allow for the selection of HPRT expressing cells from non-expressers that could be eliminated with HAT selection.

In order to check that the *gfp* and *hprt* transgenes were behaving as expected under the transcriptional control of the *flk-1* promoter, some simple differentiation experiments were carried out.

4.2.2. Results

A general differentiation protocol to check that the *gfp* transgene was behaving appropriately in both *flk-1*/GFP targeted lines was carried out. Ideally, specific differentiation protocols that maximised the direction of differentiation for *flk-1* activation would have been used however, at that stage of the project they had not been optimised. It was anticipated that some *flk-1* activation would occur in early EBs. This assumption was based on the observations made by Gordon Keller's group when developing their culture for the BL-CFC (Kennedy *et al.*, 1997; Choi *et al.*, 1998).

4.2.2.1. GFP Detection After Differentiation

EBs were generated using both suspension differentiation and the hanging drop methods (as outlined in Chapter 2) and observed daily with an appropriate GFP filter for visualisation of any GFP expressing cells.

In both the suspension and hanging drop cultures of EBs (**Figure 4.5**), patches of green fluorescent cells were seen between two and six days of differentiation. Eventually, as the EBs grew larger and developed fluid-filled cavities, true cellular fluorescence that can be focused upon using phase-contrast microscopy, could no longer be seen due to the masking effect of the high levels of autofluorescence. Autofluorescence could not be focused on, typically lit up all dense patches and any refractile, highly fluid filled cells.

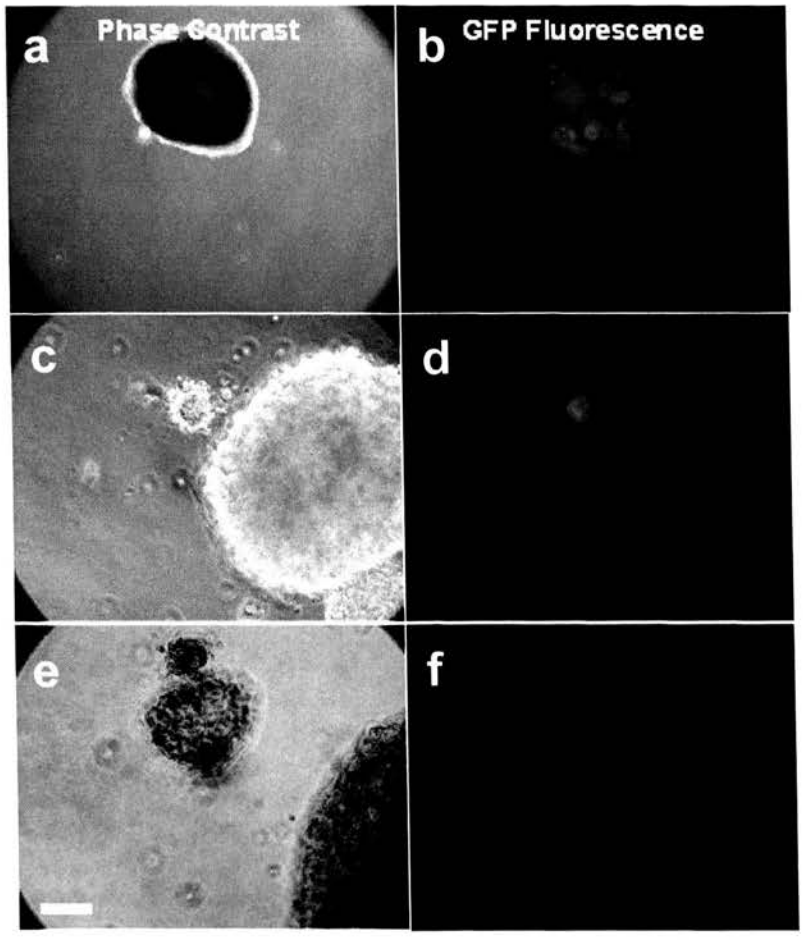

Figure 4.5 *4-day-old EBs showing GFP fluorescence.* ***a****,* ***c*** *and* ***e*** *show phase contrast images of EBs,* ***b****,* ***d*** *and* ***f*** *are the corresponding images with a GFP fluorescence filter. Scale bar represents 100μm.*

A basic haematopoietic differentiation culture in methylcellulose as well as differentiation on collagen IV were also carried out. Differentiation in methylcellulose or on collagen IV provide the basis of the two main haemangioblast culture protocols that were being optimised at the time (Kennedy *et al.*, 1997; Choi *et al.,* 1998; Nishikawa *et al.*, 1998 a and b). Growth of ES cells in these differentiation systems was attempted with the *flk-1/GFP* targeted cells to see whether, despite no optimisation of the differentiation procedures having been carried out yet, differentiation on these growth substrates would result in higher levels of GFP expression correlating with activation of the *flk-1* promoter.

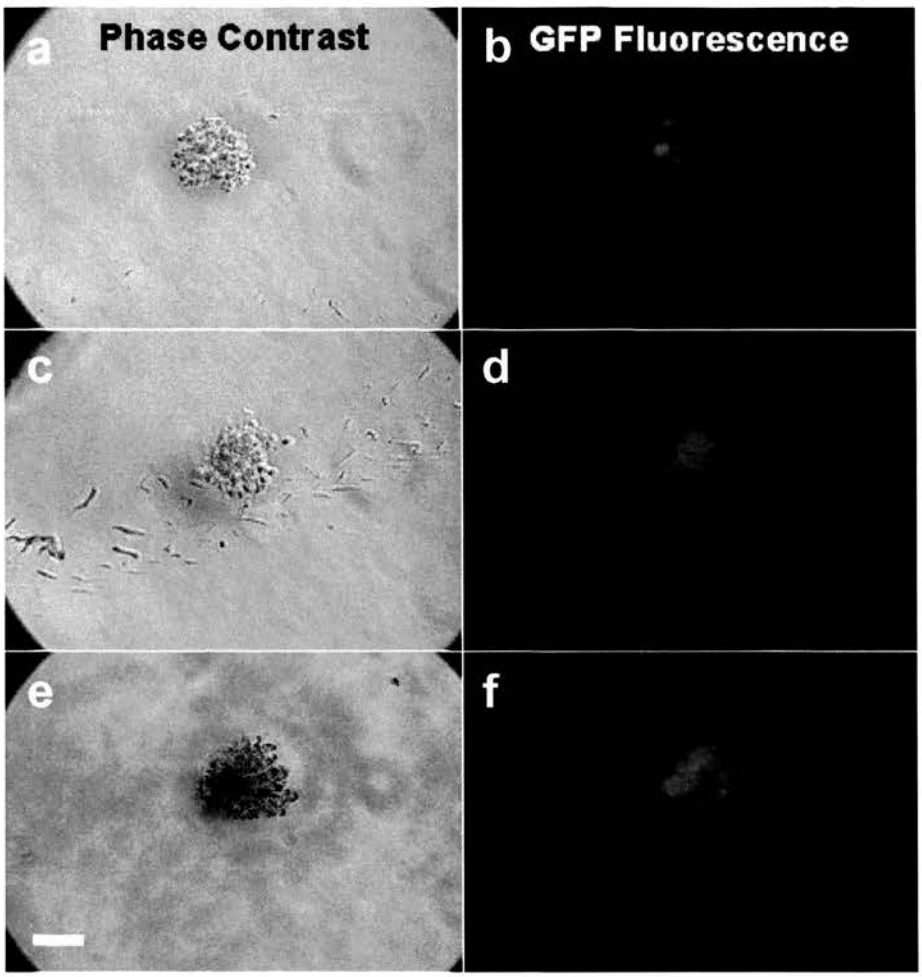

Figure 4.6 *Early colonies (day 7) from a haematopoietic culture in methylcellulose.* ***a***, ***c*** *and* ***e*** *show phase contrast images of the colonies,* ***b***, ***d*** *and* ***f*** *are the corresponding images with a GFP fluorescence filter. Scale bar shows 100μm.*

In haematopoietic cultures (**Figure 4.6**), green colonies were seen. However, unlike the EBs which typically showed patchy expression of GFP on the EB surface, in the haematopoietic cultures, colonies were generally either totally green or not green at all, with mixed colonies being the rarity. The ratio of green to non-green colonies was approximately 7:1. Non-green colonies were almost all EBs and did not have haematopoietic colony morphologies.

Cells were also differentiated on collagen IV coated plates in the absence of LIF. GFP was rarely detected visually on collagen IV differentiating cells. The cells that differentiated from the ES cells on collagen IV all possessed a high cytoplasm to

nucleus ratio with a tendency to grow as a monolayer. Most three-dimensional dense clumps, were highly autofluorescent and would not photograph well. They comprised very small, uniform, circular cells that were believed to be potential haematopoietic cells. The low level of background fluorescence observed was attributed to the high cytoplasmic volume of the differentiating cells. If GFP was being produced at relatively low levels in cells with high cytoplasm content, the GFP fluorescence might have been diluted beyond visual detection. The collagen substrate provided high levels of autofluorescence, which may have confounded GFP visualisation and masked low-level fluorescence from the cells.

4.2.2.2. Detection of Autofluorescence

An unfortunate drawback of using a GFP reporter, especially when attempting to detect it in three-dimensional structures such as EBs, was the level of autofluorescence that was observed (**Figure 4.7**).

To gauge the level of autofluorescence that could be expected from these cultures, wildtype HM1 ES cells were differentiated alongside the GFP targeted ES cell lines. Although observed at a far lower level than in the targeted lines, unexplained autofluorescence was seen in the HM1 EBs. Autofluorescence was only seen in large, dense or highly refractive, fluid-filled cells. Through the regular comparison of fluorescence between differentiating cells containing and lacking the GFP reporter, experience was gained in identifying cases where autofluorescence was a possible explanation for fluorescence according to the EB form and the size of the cells fluorescing in relation to surrounding ones that were not. In instances where such observations were insufficient to determine the authenticity of the observed fluorescence, closer examination at a higher magnification aided the identification of cases of autofluorescence.

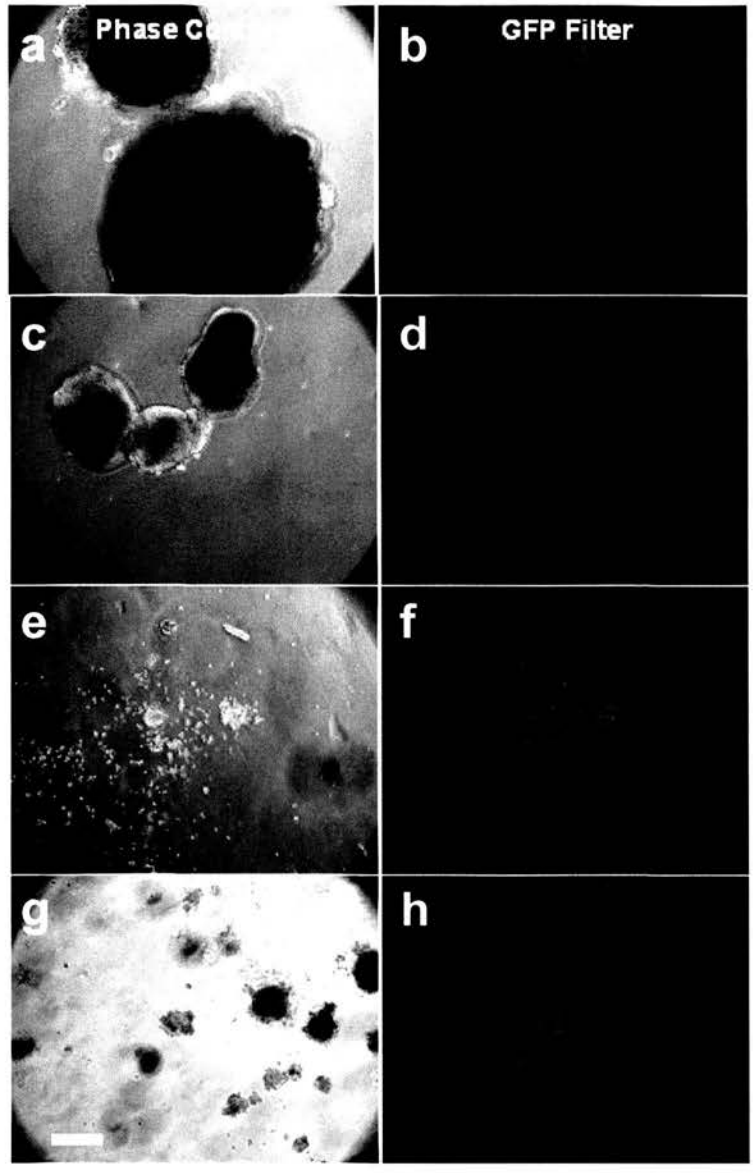

Figure 4.7 *HM1 ES cells displaying autofluorescence.* ***a***, ***b***, ***c*** *and* ***d*** *show EBs differentiated in suspension.* ***e***, ***f***, ***g*** *and* ***h*** *show colonies differentiated in methylcellulose. Scale bar represents 100μm.*

4.2.3. Loss of GFP Detection

After promising initial investigations that indicated that GFP was being expressed upon differentiation (with the assumption being that the *flk-1* promoter was activating its expression), more detailed and controlled experiments were set up to measure GFP levels at different timepoints using the various differentiation protocols under investigation both visually and via RT-PCR.

Unfortunately, GFP could no longer be detected visually in any of these experiments. Several instances of very obvious fluorescing colonies were seen, however, none that could, for certain, be distinguished from autofluorescing colonies.

4.2.3.1. Transcriptional Detection of *gfp*

Flk-1 expression, and in turn *gfp* expression may have occurred at such low levels so as not to be detectable. To see whether this was the case the D12 *flk-1*/GFP ES cell line was differentiated as EBs in suspension. RNA was obtained from pooled 5-day-old EBs and total cDNA was produced from them. Using GFP-specific primers, an RT-PCR screen was carried out to see whether the *gfp* transcript could be detected at all.

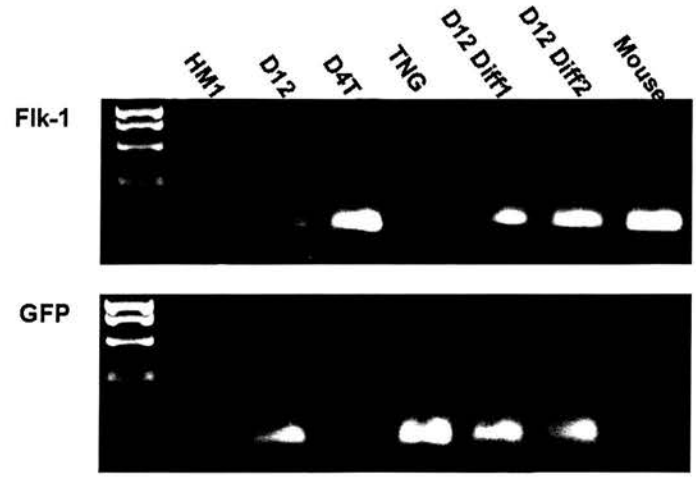

Figure 4.8 *RT-PCR showing the corresponding expression of flk-1 and gfp in the D12 flk-1/gfp targeted ES cell line against the parental* ***HM1*** *ES cell line.* ***D4T*** *is an immortalised endothelial cell line with low level flk-1 expression;* ***TNG*** *is an ES cell line that expresses GFP constitutively;* ***D12 Diff1*** *and* ***Diff2*** *correspond to two separately conducted basic differentiation experiments generating EBs which were collected for total RNA after 5 days of differentiation;* ***MOUSE*** *represents total 11 dpc mouse embryo RNA derived cDNAs.*

Figure 4.8 shows that both undifferentiated and differentiated D12 ES cells express *flk-1* and *gfp* transcriptionally. Although this PCR was not quantitative, differentiated D12 cells did appear to express *flk-1* at a higher level than undifferentiated ES cells as would be expected. This consistent result showed that the *gfp* transgene was being transcribed and that its transcription appeared to correspond to the activation of *flk-1* as expected. Reactions without reverse transcriptase were set up for all PCR reactions to ensure that no false positive results were obtained from DNA contamination (data not shown).

Although in this example, HM1 ES cells appear not to express *flk-1*, it has been observed that different populations of HM1 ES cells, like the D12 ES cell clone, can express low levels of *flk-1*. It is unclear whether this is because this ES cell line as a whole population expresses *flk-1* at low levels or whether at times it is a consequence of the cells being at a particular stage of the cell cycle, or there being a slightly differentiated cell population present that expresses it. Certainly no phenotypic differences were observed that could explain this and it appeared not to relate to the length of time in culture or passage number of the cells.

4.2.3.2. Detection of Translated GFP Protein in Targeted Cells

Although a *gfp* transcript was successfully detected in targeted cells, this was not indicative of whether the GFP protein was also being synthesised efficiently or correctly. In order to find out whether the GFP protein was being synthesised in the cells, a Western blot using total protein lysates isolated from the differentiated D12 ES cell line was carried out alongside the TNG ES cell line, which is a constitutive GFP expresser.

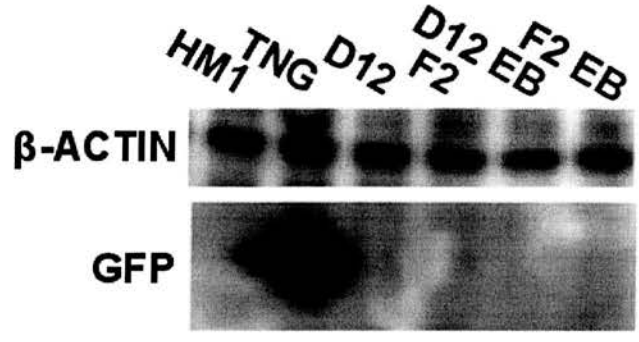

Figure 4.9 *Western blot of total protein lysates taken from GFP negative (HM1), GFP expressing (TNG) and the flk-1/GFP targeted ES lines D12 and F2. Lysates were taken from both undifferentiated cells and from 4 day old EBs.*

At first, it appeared that GFP was not being expressed in either the D12 or F2 cell lines (**Figure 4.9**), however, as the level of GFP being produced, as a percentage of the total protein content of the cells, was likely to be low in differentiated D12 and F2 cells but very high in the TNG positive control which constitutively expresses GFP under the transcriptional control of the CMV (cytomegalovirus) viral promoter, the sensitivity of this Western blot was called into question.

For a more constructive positive control, the TNG ES cell line was diluted by several orders of magnitude (1:100, 1:1000, 1:10000) with wildtype HM1 ES cells and then lysed for total protein content before the Western blot for GFP protein was repeated.

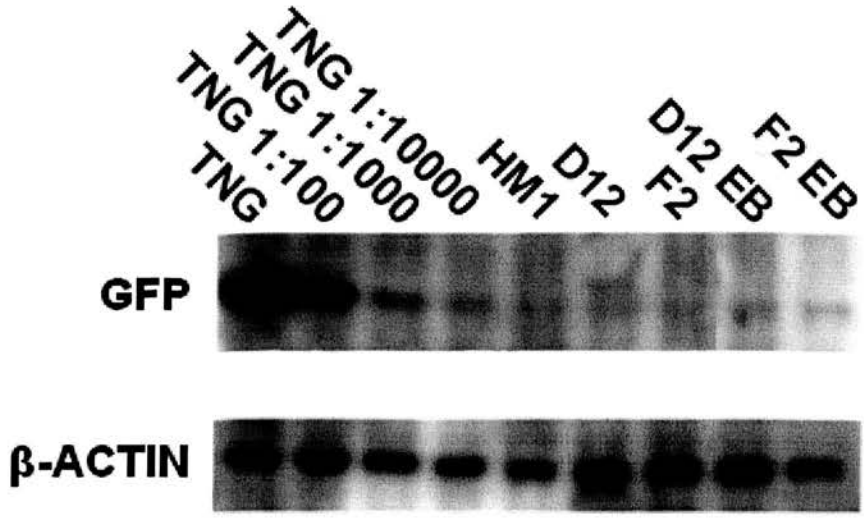

Figure 4.10 *Repeated Western blot showing dilutions of the positive control TNG line with non-expressing HM1 cells alongside the GFP non-expressing HM1 line and both the flk-1/GFP ES cell lines D12 and F2 both undifferentiated and differentiated to 4 day-old EBs.*

Figure 4.10 confirms that GFP is not being expressed at detectable levels at the protein level despite its apparent transcriptional activation. Although a background band is present in the Western blot, if it is accept that the HM1 band intensity represents no GFP protein, it is clear that neither the F2 nor D12 *flk-1*/GFP targeted lines express the GFP protein, either in the undifferentiated or differentiated states.

4.2.3.3. Tetraploid Embryo Rescue Using *flk-1/GFP* ES Cells to Detect the Expression of GFP

In order to see whether GFP could be detected in an *in vivo* setting and how the *flk-1*/GFP ES cells would behave in an environment where the *flk-1* gene is activated maximally, tetraploid embryo chimaeras were generated by Jim McWhir.

Embryos were collected at 10 dpc. At this stage *flk-1* expression in a normal embryo would be maximised due to the angiogenic remodelling of the primitive vasculature.

Embryos at this stage were also easier to dissect out of the decidua than at earlier stages of development when *flk-1* is expressed.

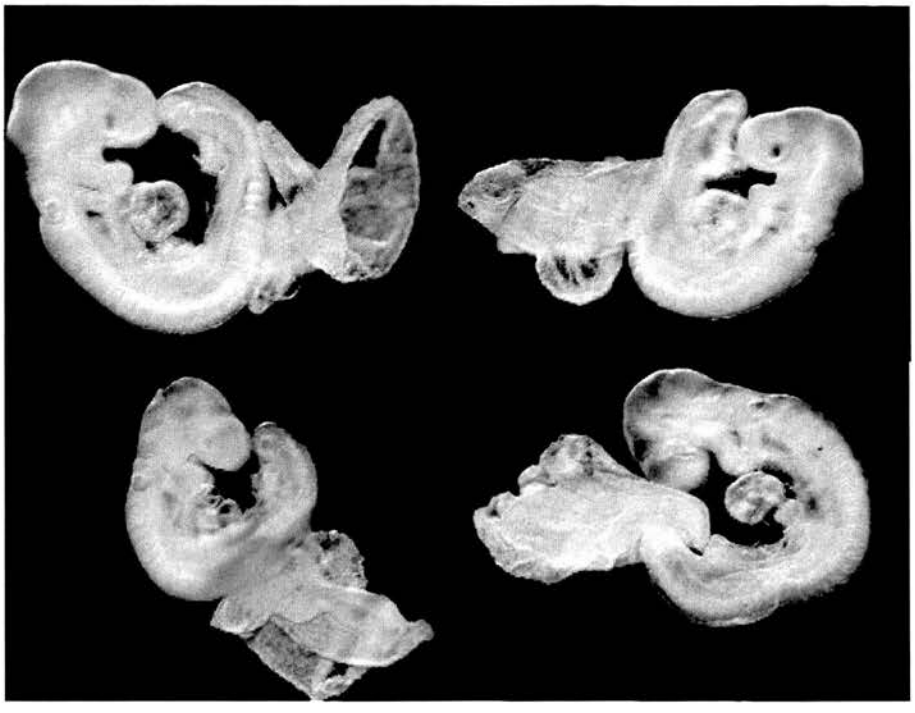

Figure 4.11 *Tetraploid embryo rescue embryos generated using flk-1/GFP ES cells. 10 embryos were successfully assessed for GFP fluorescence, however no signal could be detected for any of them.*

None of the 10 embryos analysed showed any sign of GFP expression in the vascular network or elsewhere.

4.2.3.4. Immunohistochemical Detection of GFP Expression *in vivo*

It was decided to look for GFP expression in heterozygous embryos at a stage in their development when FLK-1 would be highly expressed. The chimaeras generated from the *flk-1*/GFP ES cell line D12, were backcrossed to generate potential heterozygotes. The foetuses were removed at 11 dpc. The yolk sacs from these embryos were lysed for RNA to carry out a PCR screen (using *neo* primers) to identify heterozygotes that would be expressing *gfp* on activation of the *flk-1* gene. Only 2 out of the 10 embryos screened were heterozygotes. These embryos were cryopreserved immediately after isolation from the uterus and sectioned for staining

of the vasculature for the presence of GFP. However, in these embryos, no GFP protein was detected either visually or following immunohistochemistry for the GFP protein (in case the GFP protein was undetectable visually due to it's degradation as a consequence of the embedding and cryosectioning procedures).

4.2.4. HPRT Expression

To test the *flk-1*-specific function of the HPRT gene, the *flk-1*/HPRT ES cells were differentiated on collagen IV and placed under HAT selection to kill all $HPRT^-$ cells. Cells were seeded at a very low density as suggested by Nishikawa *et al.*, (98a and b). For the first three days of differentiation, little proliferation or differentiation was seen in any of the targeted colonies. 4 days after the differentiation regime was initiated, the cells experienced a dramatic population explosion seemingly concurrent with differentiation of cells from islands of dense ES cells.

Figure 4.12 shows the course of differentiation and the effect of HAT selection. Phenotypically, the cells that survived the selection regime were very similar to the cells from the D4T endothelial cell line. The cells were allowed to differentiate to near-confluence (7 days of differentiation) before being subjected to HAT selection. This was maintained for 3 days by which time it was clear that all the targeted cell lines were exhibiting resistance to the effects of the selection whereas the cells from the parental HM1 ES cell line had almost all died and those cells remaining appeared unhealthy and non-dividing. The targeted lines were responding to the selection, however, cells grown in the absence of HAT selection had formed a densely packed monolayer of endothelial-like cells by 10 days of differentiation.

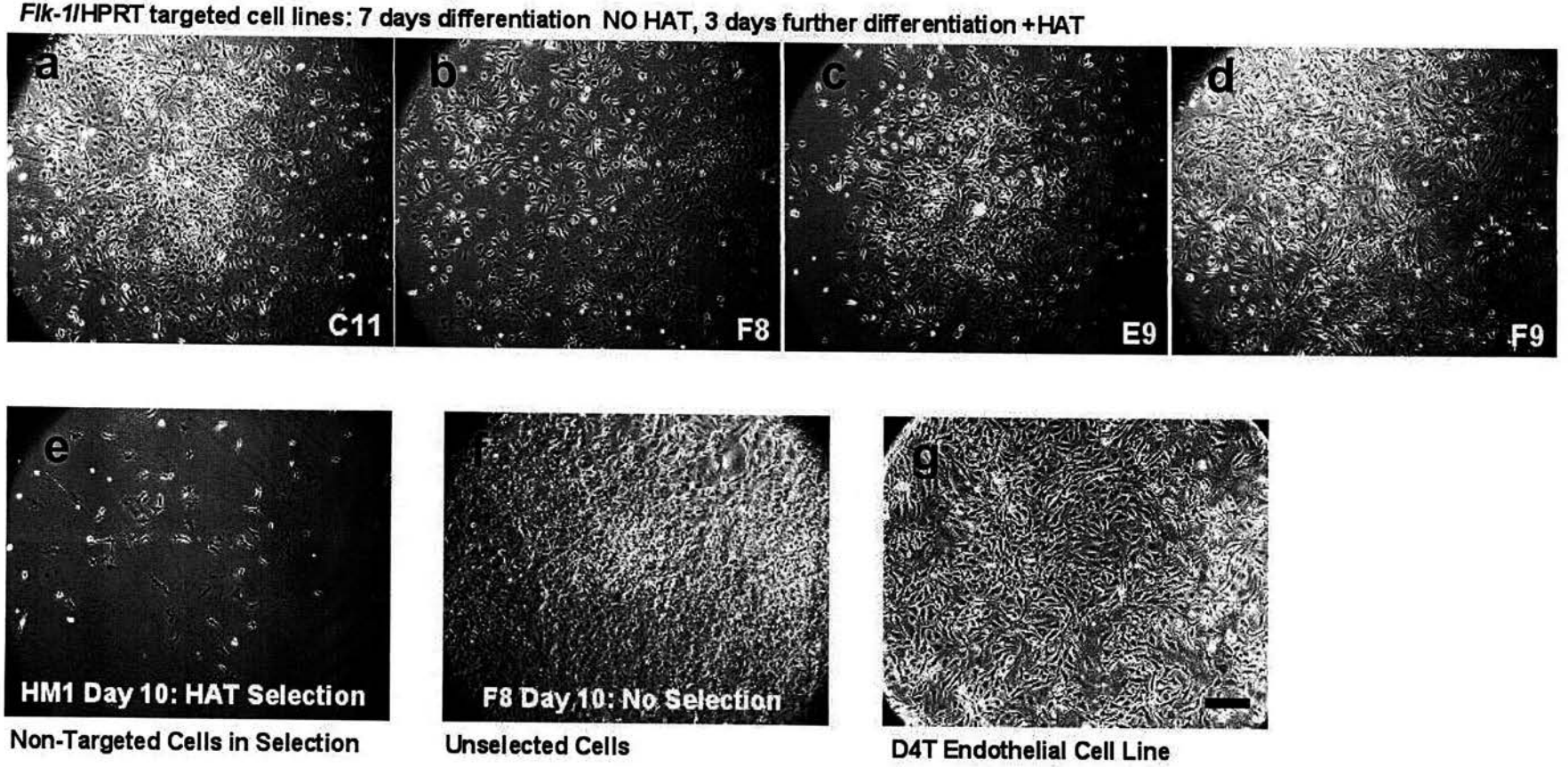

Figure 4.12 *Four flk-1/HPRT ES cell lines differentiated for 10 days on collagen IV (**a-d**). The cells were cultured in HAT selection for the last 3 days. The HM1 parental ES cell line grown under the same conditions is photographed (**e**), as well as F8 cells grown in the absence of HAT selection (**f**). The D4T endothelial cell line is included as a morphological comparison (**g**). Scale bar shows 100μm.*

To ensure that the phenotype correlated with the expression of *flk-1*, immunohistochemistry was carried out on the cells after selection was applied.

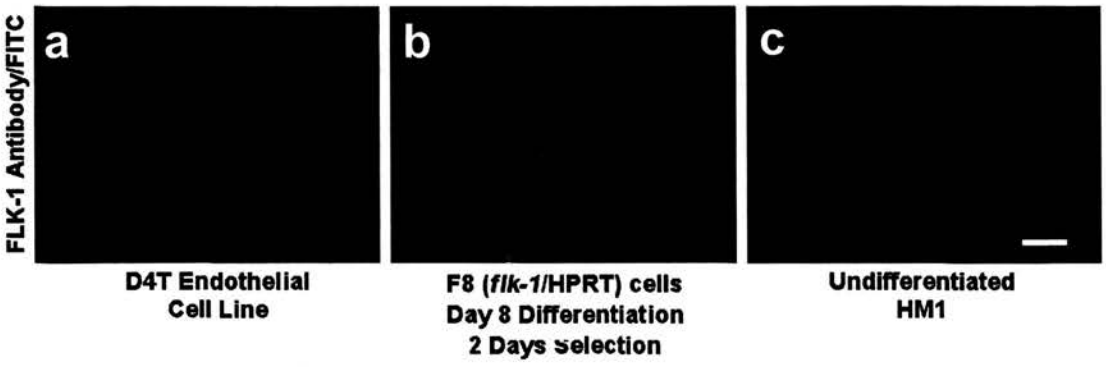

Figure 4.13 *Immunohistological detection of the FLK-1 receptor after 8 days of differentiation of ES cells on collagen IV followed by 2 days of selection (**b**). Immunohistochemical detection of FLK-1 was also carried out on the D4T cell line (**a**) and undifferentiated HM1 ES cells (**c**) as a comparison. Scale bar shows 100 µm.*

Cells in and out of selection, grown on a collagen IV substrate were found to show high levels of FLK-1 expression, comparable with D4T endothelial cells (**Figure 4.13a**). Undifferentiated HM1 cells did not stain for FLK-1 (**Figure 4.13c**).

4.3. Discussion

4.3.1. Characterisation of Targeted Lines

All of the ES cell lines that were targeted had normal chromosome counts (**Figure 4.2**). However, the clones could have acquired more subtle chromosomal mutations that would need more detailed analysis to discover. From preliminary culture and *in vitro* differentiation analyses, the F2, C11 and F9 lines were found to grow poorly and were more prone to growth crises if cultured at low densities or allowed to reach confluence. These phenomena are frequently seen in instances of single cell cloning of a cell from what is a heterogeneous population, even if the cell line in question was itself single-cell cloned. This heterogeneity manifests itself over time as a result of the accumulation of advantageous mutations that affect the growth, or morphology of a cell but which allow it to be maintained in the cell population or even overtake it (Jim McWhir, personal communication).

4.3.2. Expression of Transgene Reporters in Flk-1 Targeted Cell Lines

4.3.2.1. Flk-1/*GFP* Targeted ES Cell Lines

Following some preliminary differentiation experiments to induce *flk-1* expression, it appeared that the *gfp* transgene was being induced (**Figures 4.5, 4.6**). Care had been taken from the start to ensure that the observed instances of cell fluorescence could not be attributed to autofluorescence of three-dimensional structures in the cultures, or fluorescence from the different growth media or substrates. Follow-up experiments quickly indicated that fluorescence was no longer being observed. Attempts were made to establish whether any growth conditions had changed or whether any reagents could have been responsible for the absence of fluorescence, however, no clear reason was found.

In the initial differentiation experiments that showed GFP expression, RNA for analysis of gene expression that could have elucidated any changes at the molecular level had not been obtained. However, in later experiments in which GFP was not detectable visually, the *gfp* transcript could be detected using RT-PCR (**Figure 4.8**).

If the entire *flk-1* expressing population was expressing *gfp*, then heterochromatinisation of the modified *flk-1* locus could be ruled out as a possible cause. However, the sensitivity of PCR means that this result, indicating that *gfp* was being transcribed together with *flk-1* induction could represent a very small percentage of the total number of *flk-1* expressing cells, the remainder of which could have been subject to sufficient gene silencing to prevent the observation of green cells. The potential disruptive implications of the presence of the PGK*neo* cassette has already been discussed and is a possible cause for GFP silencing due to the induction of heterochromatinisation (Ramirez-Solis *et al.*, 1993; Rijli *et al.*, 1994; Fiering *et al.*, 1995; Olson *et al.*, 1996; Weissmann & Aguzzi, 1999) as is the process of homologous recombination itself.

Another somewhat conjectural possibility is the cytotoxic effect of high GFP expression. Although not published, this possibility has been discussed and cannot be ruled out. If a level of transcriptional regulation is lost as a consequence of the targeting event resulting in overexpression of the GFP reporter, cell death could result. Of course, this could apply to the overexpression of any protein but is more likely to be problematic if the protein is exogenously derived and not easily metabolised. It is difficult to view this possibility objectively, however, given the success of the GFP reporter in numerous examples and with GFP tagged chimaeras displaying a normal developmental course and viability. However, it is a possibility that GFP-expressing cells cycle more slowly than cells that do not express the protein due to silencing and were diminishing in the cell population over time therefore being inadvertently selected against.

Neither of the *flk-1*/GFP targeted ES cell lines produced detectable levels of GFP protein (**Figure 4.10**) *in vitro* and *in vivo* with the D12 cells having been used in tetraploid embryo complementation (**Figure 4.11**) and the generation of heterozygotes after germline transmission. The GFP reporter seemed to have been initially switched on and subsequently lost in both cell lines (although the F2 *flk-1*/GFP line never differentiated well and was not observed as closely or as frequently as the D12 line). Ideally, a FLK-1 Western blot should have also been carried out to

ensure expression of FLK-1 could be detected rather than assuming the likelihood that the levels of FLK-1 induction in EBs would be detected at the protein level. The fact that both GFP targeted clones underwent transgene silencing hinted that the GFP targeting vector design could have been responsible for the loss.

If GFP expression had truly been observed in the initial experiments and lost, this may signify some sort of instability at the *flk-1* locus brought on by the targeting, however, it would be difficult to establish what exactly occurred especially as the initial observations of GFP expression were taken visually and not corroborated by molecular analysis of some form. Others have also had success in generating *flk-1*/GFP targeted cells (Dr Alexander Medvinsky, personal communication), showing that it is possible. In this instance, a different targeting strategy and vector were used with different homologies used. The vector was constructed so that no deletion was incurred upon gene targeting. There was also no additional insertion of a polyadenylation signal for the transgenic reporter, thus relying on the capture of the endogenous polyadenylation signal.

4.3.2.2. The Effect of Targeting Vector Design on the Success of Gene Targeting

4.3.2.2.1. Deletion of a Key Regulatory Element Through Homologous Recombination

It has been shown that sequences 5' to the *flk-1* gene contain *cis*-acting promoter and enhancer elements (Ronicke *et al.*, 1996; Patterson *et al.*, 1995). These promoter elements were shown to be insufficient for expression of a *lacZ* reporter gene in murine embryos. However, when coupled with sequences from the first intron in the *flk-1* gene, the *lacZ* gene was successfully driven by the *flk-1* promoter (Kappel *et al.*, 1999). The first intron of the *flk-1* gene contains an autonomous endothelium-specific enhancer. Unlike other genes, *flk-1* lacks CAAT and TATA boxes in its promoter region that are typically responsible for controlling accurate transcriptional initiation of their corresponding genes (Ronicke *et al.*, 1996). Binding sites for GATA transcription factors and SCL/TAL1 have since been identified indicating the possible presence of a cluster of regulatory elements even though there has been no

observed influence of SCL/TAL1 on FLK-1 (Kappel *et al.*, 2000). SCL/TAL1$^{-/-}$ mice do exhibit vascular defects however (Visvader *et al.*, 1998).

Kappel and colleagues found that part of the first intron that is deleted in the targeting vector used by Shalaby *et al.* (1995) contained this regulatory element and was required for the expression of the knock-in reporter gene in the YS in later stages of vascular development and that deletion of this region reduced the expression frequency of the reporter gene. As the Shalaby construct was used in these targeting experiments, this intronic regulatory element has also been lost from all the isolated targeted lines. Shalaby and colleagues reported high levels of *lacZ* expression despite the absence of this enhancer; nonetheless, the absence of this enhancer through homologous recombination should be considered as a possible cause of the loss of GFP expression in the *flk-1*/GFP targeted cell lines.

4.3.2.2.2. The Influence of Intron Sequences on Normal Transgene Expression

A similar approach to studying potential haemangioblasts, as was used in this project, was carried out by Fehling *et al.* (2003). In this instance, the EGFP reporter was targeted to the early mesodermal marker: *brachyury*. Their intention was to isolate mesodermal cells at the earliest stage possible from differentiating ES cells via the *brachyury*/GFP reporter, sort potential haemangioblastic cells by their expression of FLK-1 and analyse the expression profile of these cells.

They used two different designs for targeting vectors. One made use of the EGFP cDNA alone; the other incorporated a splice donor site and an artificial intron before the SV40 polyadenylation sequence in order to ensure no transcription of downstream sequence taking place. A translational stop codon was also incorporated downstream of the added intron. This was to prevent the possibility of the primary transcript being recognised as an aberrant message and thus degraded as has been reported (Maquat, 2002b).

Two thirds of the first exon was deleted during targeting. The *neo* cassette was flanked by loxP sites and was subsequently removed from the targeted cell lines using Cre-mediated recombination (Gu *et al.*, 1993) to ensure that it was solely influenced by the regulatory elements of the native *brachyury* gene, and that the expression of the GFP cassette was not impacted upon by the unnecessary presence of foreign DNA sequence (Fehling *et al.*, 2003). Fehling *et al.* observed the highest expression levels of the EGFP transgene from cells transfected with the EGFP vector comprising the artificial intron and regulatory elements. This indicates that a transgene capable of undergoing post-transcriptional modifications typical of a cell may be more likely to result in it being more highly expressed as a consequence. In this study, the *flk-1*/HPRT targeting vector differed from the *flk-1*/GFP vector solely in the structure of the transgene, i.e. the EGFP transgene was a cDNA, whereas the HPRT transgene was a constructed minigene. There was a possibility that the absence of a set of coherent instructions (e.g. intron excision) for post-transcriptional modifications could lead to the loss of expression of the transgene. The importance of introns in transgene expression has been highlighted previously (Choi *et al.*, 1991; Franklin *et al.*, 1991; Brooks *et al.*, 1994).

Most mRNAs produced in eukaryotes pass through several systems that have evolved to identify and degrade aberrantly produced RNA molecules before the translation of what could potentially be mutant proteins with unknown and potentially deleterious functions. Such mechanisms exist during transcription, at the pre-mRNA processing and transport stages right up to the translation of the mRNA message (Maquat & Carmichael, 2001).

One type of mRNA surveillance is called nonsense-mediated mRNA decay (NMD) and results in decapping and degradation of mRNAs that show signs of premature termination and frameshift or nonsense mutations (Maquat and Carmichael, 2001). The process can also detect inefficient or inaccurate RNA splicing in the generation of the mRNA molecules (Maquat, 2002a).

The process of splicing induces the deposition of protein complexes at approximately 20-24 nucleotides upstream of exon-exon junctions. These complexes are called exon junction complexes (EJC). These EJCs are retained on the mRNA molecules following transfer to the cytoplasm for translation. Typically in mammals, the natural termination codon is found in the last exon. Nonsense codons, inducing premature termination are often found to occur more than 50 bases upstream of the last exon-exon boundary. If translation is terminated more than 50-55 nucleotides upstream of a junction bearing an EJC, some of constituent proteins in the EJC can initiate the NMD response resulting in mRNA degradation (Mango, 2001; Maquat, 2002b).

It is possible that the insertion of the EGFP cDNA together with its polyadenylation sequence into exon 1 could have resulted in splicing errors especially as a splice donor/acceptor site was not incorporated into the targeting vector to ensure that the cassette was not spliced out or that key regulatory elements present in the intronic sequences of the *flk-1* gene downstream to the site of insertion (Kappel *et al.*, 1999) were not spliced out by changes in the splicing pattern. This could have led to the transcription of the inserted transgenes upon *flk-1* activation but the degradation of the primary transcript before translation. This could explain why the GFP transgene was detected transcriptionally but not translationally. Also, the presence of two polyadenylation sequences may have triggered mechanisms that either affected the splicing pattern of the transcript or which caused the recognition of the transcript as aberrant, resulting in its degradation.

It is unclear if any of these factors affected the silencing of the GFP transgene in these targeted lines. It is clear however, that more care and attention should have been afforded to the initial design and construction of the targeting vectors in the initial stages of this project. The fact that both GFP-tagged cell lines lost detectable GFP expression hints that the targeting vector may have been the root of the problem. However, if this were the case, it would be expected that GFP would have been silenced or degraded and therefore undetectable from the outset. The fact that GFP was initially detected does point to there having been some sort a selective

pressure against GFP expression and for GFP silencing or loss of the GFP-expressing cells in another way.

4.3.2.3. *Flk-1*/HPRT Targeted ES Cell Lines

The expression of the selectable HPRT gene in *flk-1*/HPRT lines was more easily discerned and all four lines gave rise to HPRT-resistant cells upon differentiation. They too exhibited vastly variable behaviours as far as growth and rate of differentiation were concerned, adding emphasis to the genetic heterogeneity that exists in non-clonal populations of cells.

However, all four of the *flk-1*/HPRT targeted lines responded well to the application of HAT selection following directed differentiation for the induction of *flk-1* expression. Whereas drug selection, such as that for antibiotic resistance, can take over a week to be effective, within 48 hours of selection being applied, most non-targeted HM1 parental cells had been eliminated and only endothelial-like cells remained in the differentiation cultures of the targeted cell lines (**Figure 4.12**).

When such cultures were fixed and stained for the expression of FLK-1, patchy expression was seen in differentiation cultures that were not in selection. Cells surviving HAT selection all stained positively for FLK-1 provisionally indicating that the HPRT transgene was behaving as desired at the *flk-1* locus.

Further optimisation of *flk-1*/HPRT cells to isolate a pure progenitor population is investigated in Chapters 5 and 6.

4.4. Summary

- The 4 *flk-1*/HPRT and 2 *flk-1*/GFP targeted ES cell lines were found to be variable with respect to proliferation, differentiation and vigour as a consequence of single-cell cloning but were all karyotypically normal.

- The pluripotency of the most vigorous clone from each reporter, D12 (*flk-1*/GFP) and F8 (*flk-1*/HPRT), were tested with the generation of tumours in SCID mice. Tissues from all three germ layers were observed in the tumours. Additionally, the D12 ES cell line was successfully used in the generation of chimaeric mice.

- The GFP reporter was found to be silenced or inactive although the cause was undetermined.

- The HPRT reporter was confirmed to be functional and successfully used to select for a cell population with endothelial cell morphology.

- The *flk-1*/HPRT lines will be used to optimise the isolation of a FLK-1 expressing progenitor population capable of contributing to the vascular lineages upon *in vivo* engraftment.

CHAPTER 5

Directed Differentiation for Flk-1 Expression I: Keller Protocol

Directed Differentiation for Flk-1 Expression I: Keller Protocol

5.1. Introduction: Haemangioblast Differentiation

The differentiation of "blood" from ES cells is one of the most studied differentiation programmes. It is not a rarity to see patches of red cells on cystic embryoid bodies grown in suspension, with only LIF having been omitted from the ES cell growth medium, and with no stimulatory factors having been added (**Figure 5.1**). However, to differentiate a definitive haematopoietic cell rather than the primitive erythroid cells seen in embryoid bodies, several stages of differentiation have to be carried out, typically incorporating the addition of growth factors.

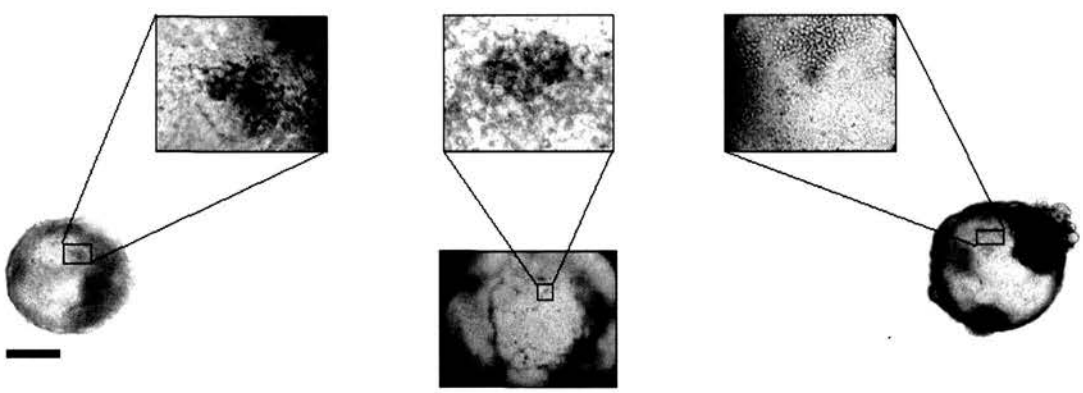

Figure 5.1 *Cystic EBs formed from simple suspension cultures of ES cells in the absence of LIF. Scale bar represents 1mm. The erythroid patches observed are magnified x10.*

A 2.5-3.5-day-old EB contains cells that are believed to be haemangioblastic and can be harnessed to differentiate to cells of the haematopoietic system (Kennedy *et al.*, 1997; Choi *et al.*, 1998). Beyond this window of differentiation, other signalling events and pathways will emerge and predominate as the receptor profile of the differentiating cellular population changes and the potential to respond to haematopoietic ligands is lost.

5.1.1. Three-Dimensional Differentiation on Methylcellulose

The methylcellulose culture regime devised for the culture and differentiation of cells with haematopoietic potential (Wiles and Keller, 1991) has been modified for the culture of progenitors with the capability to differentiate to endothelial as well as haematopoietic cells (Keller *et al.*, 1993; Kabrun *et al.*, 1997; Kennedy *et al.*, 1997; Faloon *et al.*, 2000; Robertson *et al.*, 2000; Fehling *et al.*, 2003). It had been found that EBs generated in suspension culture, had the largest potentially haemangioblastic cell population (as measure by the number of FLK-1^+ cells) at 2.75 days in the 24-hour window of their existence in EBs (Kennedy *et al.*, 1997). This modified culture regime involves the propagation of 2.75-day-old EBs, their disaggregation and replating of a defined cell number in a methylcellulose mix containing high levels of the growth factors Stem Cell Factor/c-KIT ligand and VEGF.

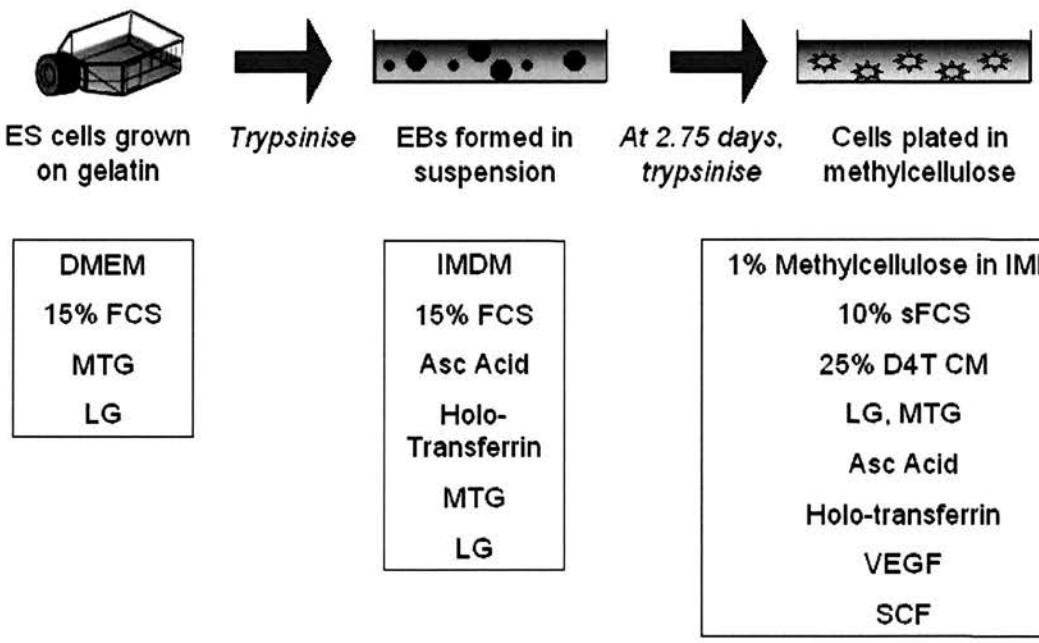

Figure 5.2 *The Keller protocol for the isolation of haemangioblastic progenitor colonies. The diagram shows the basic procedure for differentiation. The boxes show the components of the growth media involved at each stage. MTG, monothioglycerol; LG, L-glutamine; Asc Acid, ascorbic acid;* ***sFCS****, FCS tested for its ability to support haematopoietic differentiation; D4T conditioned medium, conditioned medium from the D4T endothelial cell line.*

The protocol shown in **Figure 5.2** was developed by Gordon Keller and his research team (Wiles and Keller, 1991; Keller *et al.*, 1993; Kabrun *et al.*, 1997; Kennedy *et al.*, 1997; Faloon *et al.*, 2000; Robertson *et al.*, 2000; Fehling *et al.*, 2003).

Wiles and Keller found that by culturing ES cells in a three-dimensional methylcellulose matrix, the growth and differentiation of haematopoietic colonies from differentiating ES cells was far better supported than haematopoietic differentiation of EBs in suspension culture. Erythroid cells in growing colonies were seen after 7-8 days in culture. By 10 days, 40-60% show signs of erythrogenesis and the colonies rupture to release the erythroid cells in the periphery. Further investigation into the effects of added supplements led to discoveries that ascorbic acid acted as a reducing agent and increased the number of macrophage colonies seen, erythropoietin increased the number of erythroid cells differentiating and IL3 increased the number of erythroid cells associating with EBs and prolonged their survival. IL3 also increased the appearance of macrophage-like colonies and affected the kinetics of their appearance as they appeared 2 days earlier than they would in the absence of IL3 (Wiles and Keller, 1991).

Initially, only erythroid and basic haematopoietic colonies were obtained from methylcellulose cultures (Wiles and Keller, 1991) however the protocol was refined and elaborated to enable the propagation of more complex haematopoietic progenitor types (Kennedy *et al.*, 1997). Incorporated into the protocol was an initial step when EBs were generated in suspension prior to disaggregation and plating in methylcellulose. In the haematopoietic cultures, "blast" morphological colonies were identified and were distinguishable from secondary EBs, which had far denser structures. The blast colonies were identified when 3-3.5 day old EBs were disaggregated and plated in methylcellulose with KL and VEGF. When picked after 4-6 days from this methylcellulose-based medium and replated in methylcellulose medium supplemented with haematopoietic cytokines, many different haematopoietic lineages were observed including both primitive and definitive haematopoietic colonies (Kennedy *et al.*, 1997).

Kennedy and colleagues found that the best growth of blast colonies was achieved when the cultures were set up with conditioned medium obtained from an EB-derived endothelial cell line called D4T (Williams *et al.*, 1988). It was also observed that an increase in the frequency and size of blast colonies was dependent on the inclusion of kit-ligand and VEGF in the cultures along with the synergistic influence of the D4T conditioned medium but that omitting the growth factors rendered most of the plated cells unresponsive to the effects of the conditioned medium (Kennedy *et al.*, 1997). The added growth factors must therefore have had an inductive influence on the cells, priming them for differentiation to haematopoietic lineages and rendering them responsive to the elements in the conditioned medium that augmented their potential to form blast colonies. Individual blast colonies were found to be able to form cells from a variety of haematopoietic lineages.

Blast colonies did not express *brachyury* whereas EBs did, indicating that EBs were from an earlier developmental stage. Many blast colonies expressed both primitive and definitive globin genes. All blast colonies expressed FLK-1, CD34, SCL/TAL1 and GATA1.

The necessity for VEGF in the isolation of BL-CFC to stimulate the expression of the endothelial marker *flk-1*, suggested that the BL-CFC might also have had endothelial potential. This was confirmed by Choi *et al.* (1998) who showed that the BL-CFC was clonal and had the ability to differentiate to both the haematopoietic and endothelial lineages. A haemangioblast differentiation experiment was carried out using a mixture of two different cell populations, each bearing a different antibiotic selection cassette. Individual haemangioblastic blast colonies were further differentiated down both endothelial and haematopoietic lineages. It was found that endothelial and haematopoietic cells derived from the same blast colony always had a common selection gene expressed. Coexpression rarely occurred and was attributable to contamination (Kennedy *et al.*, 1997; Choi *et al.*, 1998).

These observations led to the belief that the BL-CFC represented the *in vitro* equivalent of the haemangioblast with the same bipotentiality to differentiate further

down both the haematopoietic and vascular lineages (Choi *et al.*, 1998). Further work has since shown that this basic culture can be enhanced with the addition of bFGF and activin A that have a synergistic influence on the efficiency of blast colony formation (Faloon *et al.*, 2000) with bFGF stimulating the proliferation of haemangioblasts and VEGF inducing their maturation (and migration *in vivo*).

5.1.2. Stepwise Conversion of a Haematopoietic Culture to the BL-CFC Culture Regime

Many attempts to replicate the BL-CFC culture protocol were carried out without success (data not shown). The following experiments describe the steps initiated to convert a basic haematopoietic colony culture to the haemangioblast-specific BL-CFC culture regime. Individual variables were modified in basic haematopoietic cultures using disaggregated EBs to adjust the haematopoietic methylcellulose culture to the full BL-CFC culture regime by introducing individual elements of the BL-CFC culture to the methylcellulose in steps.

Success of the differentiation regimes were assessed from 7 days post-plating by counting the total number of colonies present in each 35mm dish as well as scoring the colonies for signs of differentiation to colonies typically seen in a haematopoietic culture.

5.1.2.1. Comparison of Different Disaggregation Techniques on the Viability of EB-Derived Cells used in Methylcellulose Cultures

Initial experiments suggested that cell viability after disaggregation was affecting the success of the methylcellulose cultures. Two different disaggregation enzymes were therefore compared for their effect on cell viability: TEG, a trypsin based disaggregation solution that had been used until then for all disaggregation steps in the culture of cells and dispase. Dispase is a bacillus-derived neutral metalloprotease that is believed to have an effective but much more mild proteolytic action than trypsin. It is a highly selective protease and cleaves fibronectin, collagen IV, and to a lesser extent collagen I, but not collagen V or laminin. Trypsin is a serine

peptidase that preferentially cleaves at lysine and arginine residues and is therefore more indiscriminate than dispase. Bearing in mind that the EBs would have to be exposed to the proteases for a longer period of time than a monolayer would be to obtain a good single-celled suspension, it was conceivable that prolonged exposure to trypsin, coupled with the added mechanical disaggregation that is necessary to break up three-dimensional EBs, could result in cell viability and growth being affected by the disaggregation technique.

5.1.2.1.1. Experimental Outline

Parallel experiments were set up to compare trypsin disaggregation and dispase disaggregation. Both 3 and 4-day-old EBs were generated from four different ES cell lines (one *flk-1*/GFP cell line, one *flk-1*/HPRT cell line, the parental HM1 line and the non-parental R1 ES cell line) and disaggregated, using either trypsin or dispase. 1000 viable disaggregated cells were placed in haematopoietic cultures in methylcellulose. The methylcellulose plates were examined after 7 days and again after 14 and 21 days culture and assessed for the number of growing colonies as well as their differentiation status by scoring the colonies according to their morphology as shown in **Figure 5.3**.

5.1.2.1.2. Results

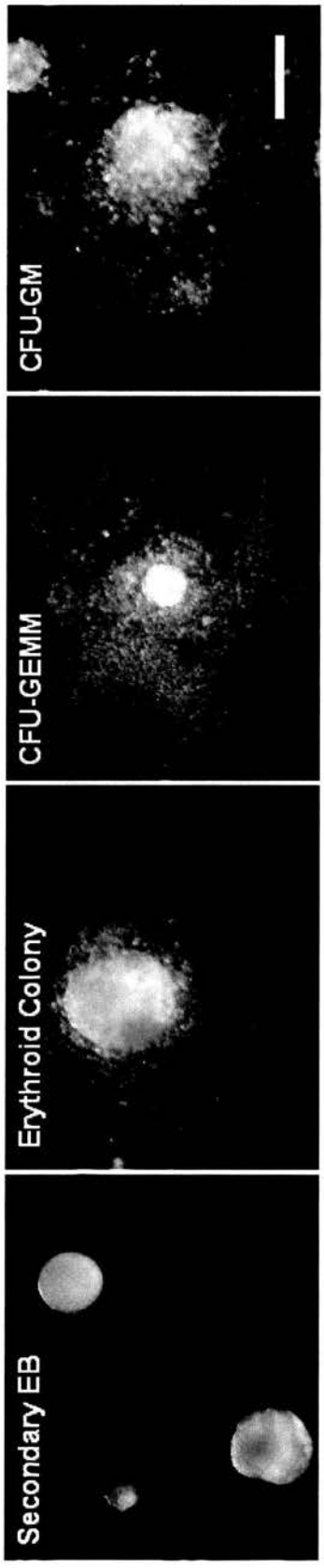

Figure 5.3 *Common colony types observed in haematopoietic cultures in methylcellulose after disaggreagation of 3-4 day old EBs. Secondary EBs are the most primitive; erythroid colonies, CFU-Granulocyte, Erythrocyte, Monocyte, Macrophage (**CFU-GEMM**) and CFU-Granulocyte, Macrophage (**CFU-GM**) are all more mature haematopoietic colony types. Scale bar represents 1 mm.*

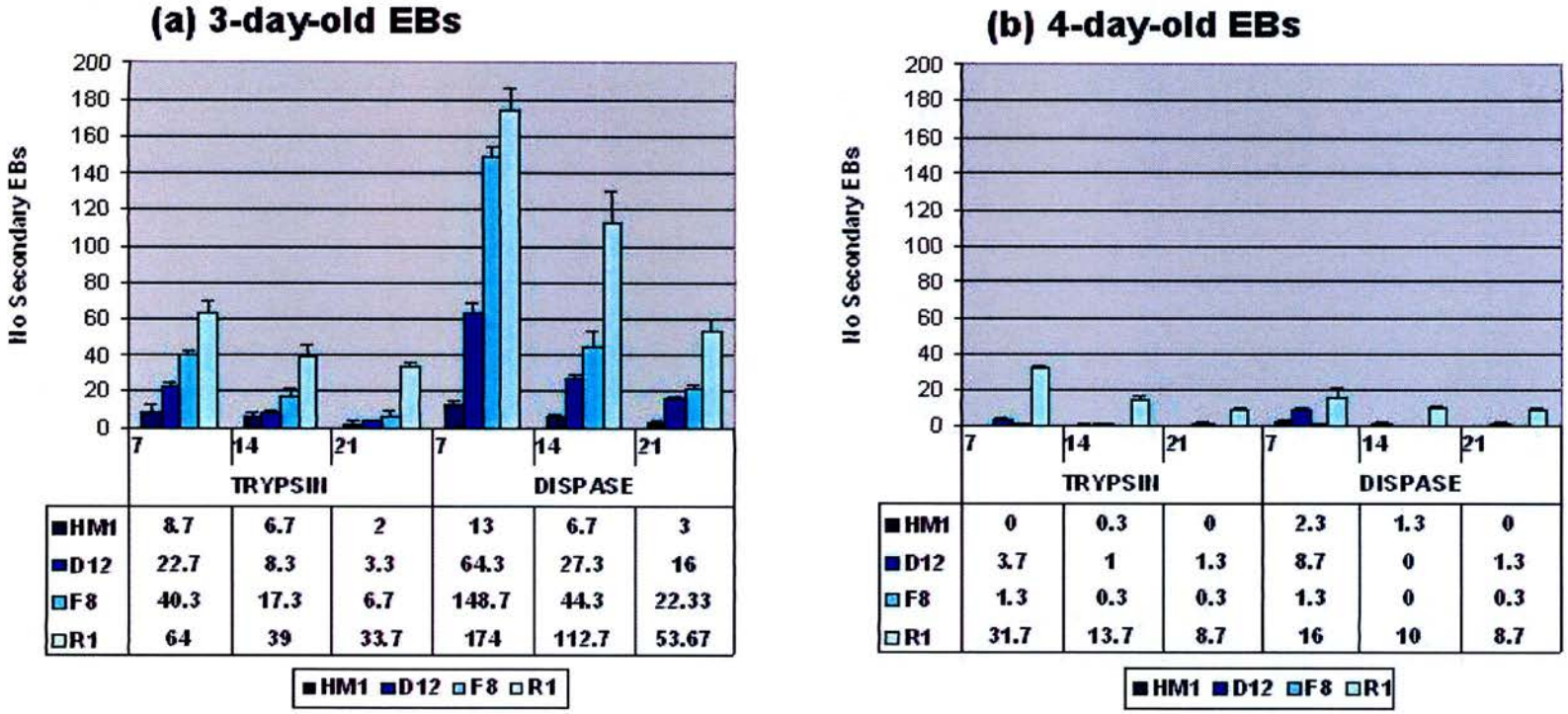

Figure 5.4 *Bar charts showing the mean number of secondary EBs observed in methylcellulose cultures of (**a**) 3 and (**b**) 4 day old EBs disaggregated with either trypsin (TEG) or dispase at 7, 14 and 21 days post-disaggregation. Three replicates were observed for each ES cell line. Bars represent the mean of 3 replicates. Error bars represent the standard error.*

The data presented in **Figure 5.4** show that the dispase treatment resulted in a higher yield of secondary EBs forming in the methylcellulose cultures than the trypsin disaggregation when 3-day-old EBs were used in the culture regime. 4-day-old EBs showed very low haematopoietic capabilities in comparison, and no conclusion with respect to the disaggregation techniques can therefore be made. Analysis of variance was used to look at the effects of treatment (trypsin and dispase), cell line (HM1, R1, D12, F8) and day (7, 14, 21) on the numbers of EBs observed. For day 3 EBs dispase disaggregation yielded significantly more EBs than trypsin disaggregation ($p<0.001$) and the number fell significantly from 7 to 14 and then to 21 days ($p<0.05$) as the EBs differentiated to haematopoietic colonies. The R1 cell line produced the most EBs followed by F8, D12 and HM1. The differences between individual lines were significant ($P<0.05$). For day 4 EBs the interactions between treatment, day and cell line are all significant and no general statements can be made about the significance of these effects. The trend is for numbers of EBs to decrease as day increases from 7 to 21 and for R1 to produce the highest numbers of EBs, as was the case for day 3 EBs. There is no clear difference between trypsin and dispase, unlike day 3 EBs, probably due to the overall paucity of secondary EB generation from 4-day-old EBs.

The paucity of older EBs to form haematopoietic colonies supports the observation of Kennedy *et al.* (1998) that the window for differentiation down the haematopoietic lineage (more specifically, the haemangioblastic lineage) is early and limited to a 24-hour window between 2.5 and 3.5 days after EBs are generated. These data also show an interesting level of variation between the different ES cell clones. The R1 ES cell line was seen to be the most vigorous. R1 ES cells grow faster than HM1s when being maintained in a pluripotential state (personal observations) and interestingly, the HM1 parental ES line was the poorest performer in all the haematopoietic cultures carried out, yielding far fewer colonies than the D12 and F8 lines. This was likely to be due to the HM1 line being a heterogeneous cell population, whereas the targeted lines were clonally isolated from it, thus bearing slightly different growth characteristics.

At each time interval, the number and type of haematopoietic colonies observed were noted. After 7 days of culture, only secondary EBs were observed, however, over time the total number of secondary EBs declined as the EBs became more differentiated and mature haematopoietic colonies emerged (**Figure 5.5**).

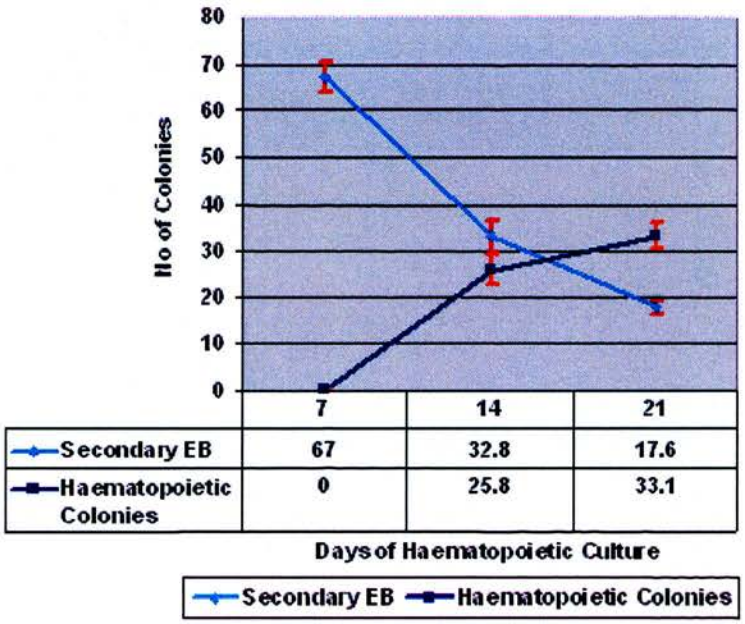

Figure 5.5 *The means of all secondary colonies and all haematopoietic colonies over a 3-week observation period. Data points on line graph represent the mean of 6 replicates. The data for both trypsin and dispase dissociation were combined. Standard error bars are shown in red for secondary EBs and orange for haematopoietic colonies.*

The raw data showed that there was a high level of variability in the colony yield in methylcellulose and that only a small percentage of cells plated were surviving to proliferate and differentiate to form colonies. This was in part typical of methylcellulose colony cultures and also partially due to the harsh nature of the disaggregation methods used. To disaggregate EBs, the exposure to both the proteases had to be prolonged in comparison to the typical incubation time required to disaggregate cells growing as a monolayer, which is 1-3 minutes. The exposure to dispase was 1 hour, after which time, the EBs had to be disaggregated mechanically.

4-day-old EBs were beyond the time frame for maximal haematopoietic differentiation as was seen from the general poor yield of secondary EBs (**Figure 5.4**). It was decided to carry out the dispase and trypsin disaggregation methods using 2.75 and 3.5 day old EBs. If the HM1 ES line and its derivatives behaved as the CCE ES cells that the Keller group had used to optimise this protocol, the 2.75-day-old EBs would be expected to differentiate into haematopoietic lineages more successfully than the older 3.5-day-old ones. Apart from the age at which the EBs were disaggregated, the plating experiment was carried out as the previous one.

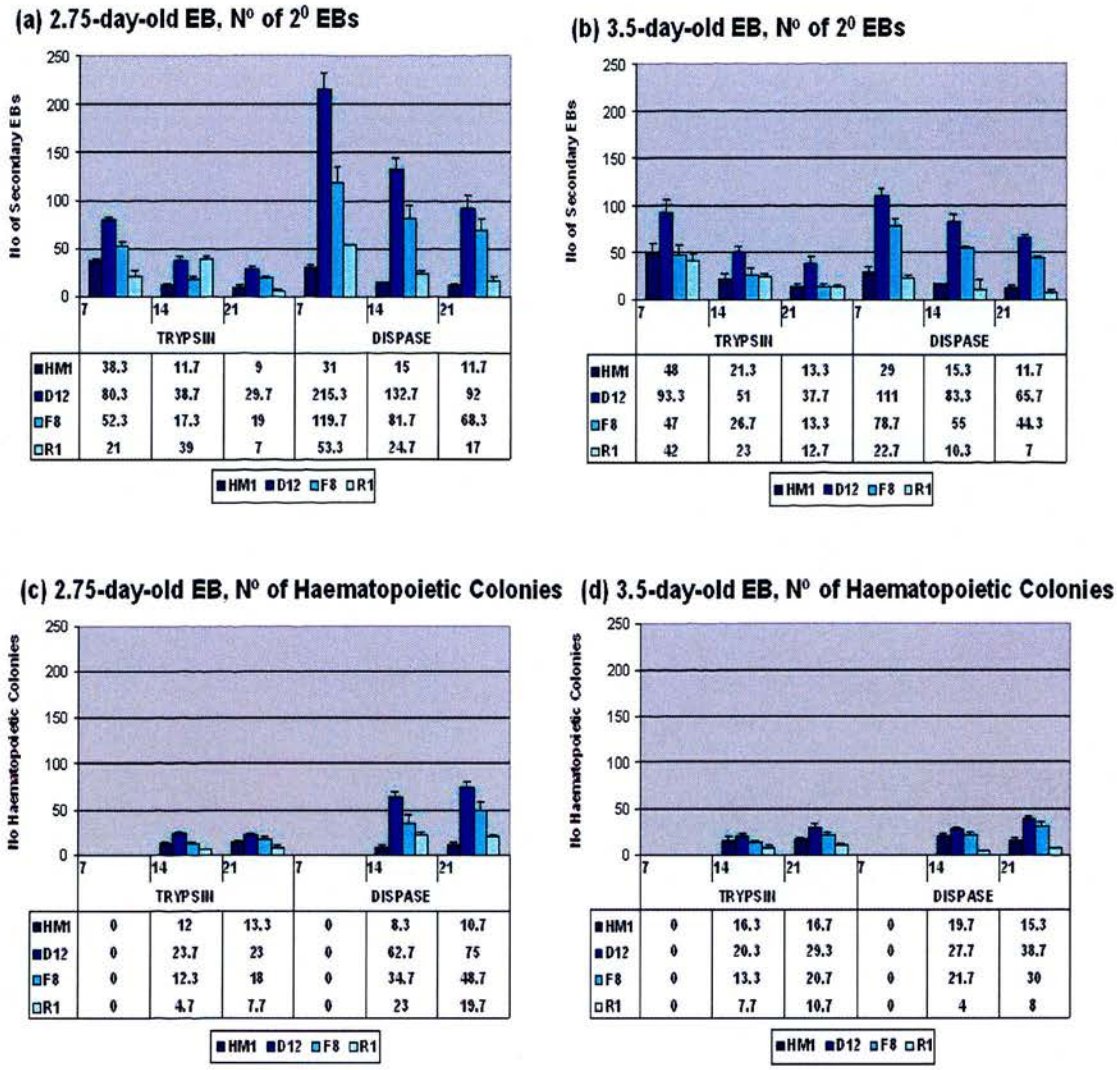

Figure 5.6 *Haematopoietic culture after either trypsin and dispase disaggregation of 2.75-day-old (**a, c**) and 3.5-day-old (**b, d**) EBs. The mean average of three replicates of each variable were calculated for each ES cell clone and plotted against each other. Figures **a** and **b** show the number of secondary EBs seen in the haematopoietic cultures after 7, 14 and 21 days in the methylcellulose. Figures **c** and **d** show the number of differentiated haematopoietic colonies that were observed in the cultures at the same time points. Bars represent the mean of 3 replicates. Error bars show standard errors.*

For 2.75-day-old EBs there was a significant interaction between the method of disaggregation and the cell line. This is due to the fact that HM1 parental cell line has almost the same number of EBs under trypsin disaggregation and dispase disaggregation compared with R1, D12 and F8, which yielded more secondary EBs when initial EB preparations were disaggregated treatment than with trypsin

disaggregation. The effects of treatment and day are both significant ($p<0.01$). Dispase is superior to trypsin disaggregation and the numbers of EBs decreases approximately linear as the number of days in differentiation increases from 7 to 21.

For 3.5-day-old EBs there is a significant interaction between treatment and cell line. There is no consistency between the treatments. Trypsin disaggregation was superior for F8 and HM1 cell lines, dispase was superior for R1 and D12, which yielded higher numbers of EBs on both treatments than the other 2 cell lines. If you do not look at the data from each cell line individually, there was no statistically significant difference between the two treatments. However, given the low number of secondary EBs generated from 3.5-day-old EBS, the

Figure 5.6 shows data that supports previous observations that dispase disaggregation has an influence on the viability of cells and in turn the efficacy of methylcellulose haematopoietic cultures. However, it was found that individual cell lines did respond differently to the disaggregation methods tested. EBs from the F8 and HM1 cell lines did not yield more secondary EBs when disaggregated with dispase, however this was likely due to the fact that both these lines responded poorly to the differentiation protocol and that the method of disaggregation of F8 and HM1 EBs was overridden by this poor response. Overall, these data do indicate that dispase is a better enzyme for disaggregating the EBs for methylcellulose differentiation.

When the cells were being counted after disaggregation, the dispase treated samples often had a noticeably large amount of cell debris, presumably from lysed cells. It was therefore assumed that the treatment was very harsh which makes this result all the more surprising. Although the trypsin treated cells appeared healthier when counted, their viability over a longer period of time may have been affected resulting in cell death after the set-up of the cultures. It was decided to use dispase to disaggregate the EBs from that point forth. By replacing the PBS used to dilute the dispase with IMDM base medium (see Chapter 2 for the procedure), the level of cell lysis was also reduced.

These data (Figure 5.6) again showed that earlier EBs have a greater potential for haematopoietic differentiation than more differentiated ones. It seems that the HM1-derived targeted ES cell lines have more haematopoietic potential at 2.75 days than older EBs and could have a similar potentiality within the 24 hour window for haematopoietic and haemangioblastic differentiation suggested by the Keller group (Kennedy *et al.*, 1997). Dispase disaggregation of EBs yielded significantly more haematopoietic colonies than trypsin disaggregation when the variation between cell lines was omitted from the analysis ($p<0.01$). See **Appendix B** for the full statistical analyses of these data.

5.1.2.2. Addition of D4T Conditioned medium

The next step towards optimising the methylcellulose culture for BL-CFCs was to add the D4T conditioned medium and see what effect it had on the growth of colonies in the methylcellulose. It was possible that the disaggregation method had not been the only reason for the failure of the BL-CFC cultures, it was also considered possible that the preparation of the D4T conditioned medium was either toxic, thus preventing growth, or the D4T cells had not conditioned the medium sufficiently to allow for the differentiation of BL-CFC with the recommended concentrations of the added SCF and VEGF.

5.1.2.2.1. Experimental Outline

Previously when the BL-CFC culture had been carried out, either 1 or 3 day-old conditioned medium had been generated by placing the standard culture medium for D4T cells minus the ECGS onto the cells for the appropriate length of time. The cells were only used for conditioning once and the medium was used fresh from the cells after filtration. To test for the efficacy of different preparations of D4T, 3 different preparations were compared to 3-day-old conditioned medium from a previous experiment that had been frozen. The conditioned medium was added to the basic haematopoietic differentiation medium at the BL-CFC culture recommended concentration of 25% (v/v).

Old (frozen CM) 3 days conditioning medium –ECGS

1 day conditioning medium –ECGS

3 day conditioning medium –ECGS

3 day conditioning medium +ECGS

The D4T cells were in this instance used at high confluence. Three replicates with 1000 cells in each using dispase disaggregation of 2.75-day-old EBs were plated for each medium type and left for 10 days before a colony count was carried out.

5.1.2.2.2. Results

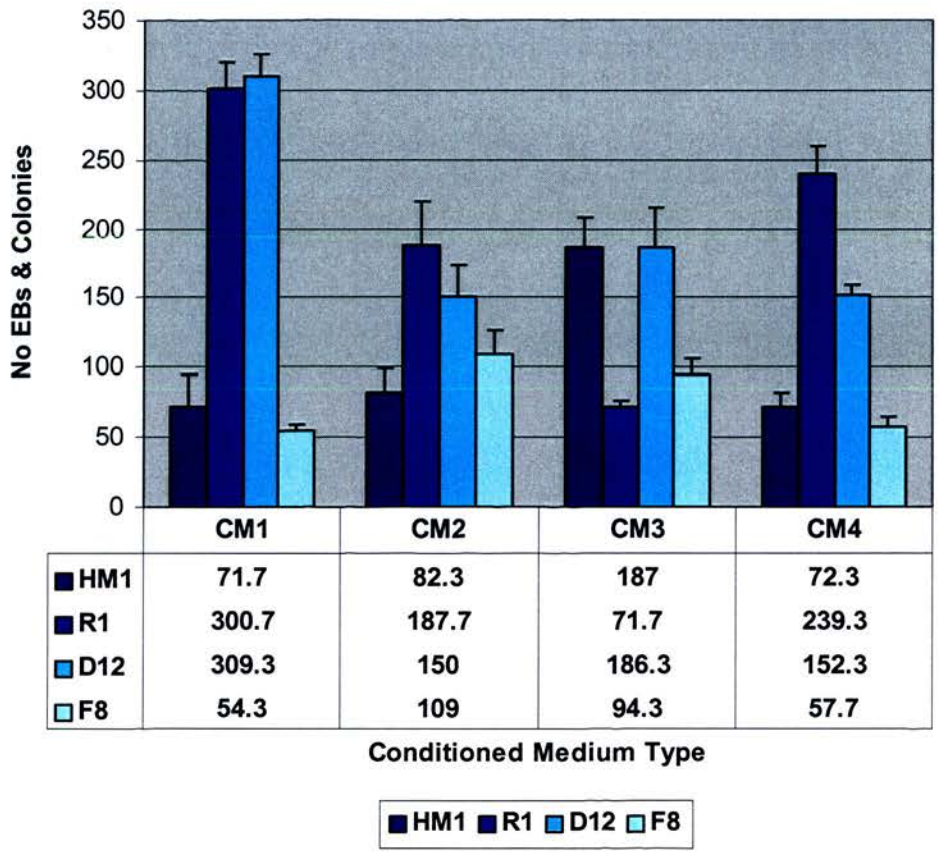

Figure 5.7 *Bar charts of the proliferative effect of different preparations of D4T conditioned media on ES cells in basic haematopoietic cultures in methylcellulose. CM1, old 3 day CM; CM2, 1 day conditioning –ECGS; CM3, 3 days conditioning –ECGS; CM4, 3 days conditioning +ECGS. Bars represent the mean of 3 replicates. Error bars show standard error.*

Statistically there is no significant difference between the different media and it is not easy to decide which one is best in any sense. CM1 has the highest overall average (the other 3 have very similar overall averages) and maintains R1 and, D12 cells well but supports HM1 and F8 differentiation cultures. CM4 is perhaps the worst because it does fairly badly for all the cell lines except R1.

If a judgement is made based on the relative performance of the media for a subset of the cell lines i.e. R1 and D12 (given that these cell lines were more resilient in all previous comparisons), then CM1 better.

These results therefore indicated that the old, frozen preparation of D4T conditioned medium was marginally better overall at increasing the survival and colony formation of the plated cells in the haematopoietic cultures (**Figure 5.7**). However, as this conditioned medium was the same as "CM3" (freshly prepared 3-day conditioned medium in the absence of ECGS), the variability can be attributed to the better conditioning state of the D4T cells when the old conditioned medium was prepared, or it may be due to the inherent variability of the methylcellulose cultures.

An interesting possibility arises when it is considered that the old conditioned medium had been frozen and thawed for use in this experiment whereas the other samples were fresh preparations. The freeze-thaw process may have resulted in the degradation of inhibitory growth factors that could otherwise result in diminished or retarded growth of colonies in the methylcellulose culture medium.

As the different preparations of conditioned medium did not have a significant bearing on the success of the haematopoietic cultures. It was decided to use the 3-day conditioning regime in the absence of ECGS for the BL-CFC cultures.

5.1.3. Identification of the BL-CFC

After having ensured that the disaggregation method and the added conditioned medium were as favourable as possible, the published conditions for the BL-CFC culture regime were tested (Kennedy *et al.*, 1997; Choi *et al.*, 1998).

The BL-CFC culture was set up using 2.75-day-old EBs disaggregated using dispase and plated at 1000 cells per 35mm dish. The optimisation procedures were eventually successful in rectifying the underlying problem with getting this culture system to work and eventually, colonies that matched the description provided of the BL-CFC (Kennedy *et al.*, 1997; Choi *et al.*, 1998; Faloon *et al.*, 2000; Robertson *et al.*, 2000; Kennedy and Keller, 2003) were observed (**Figure 5.8**).

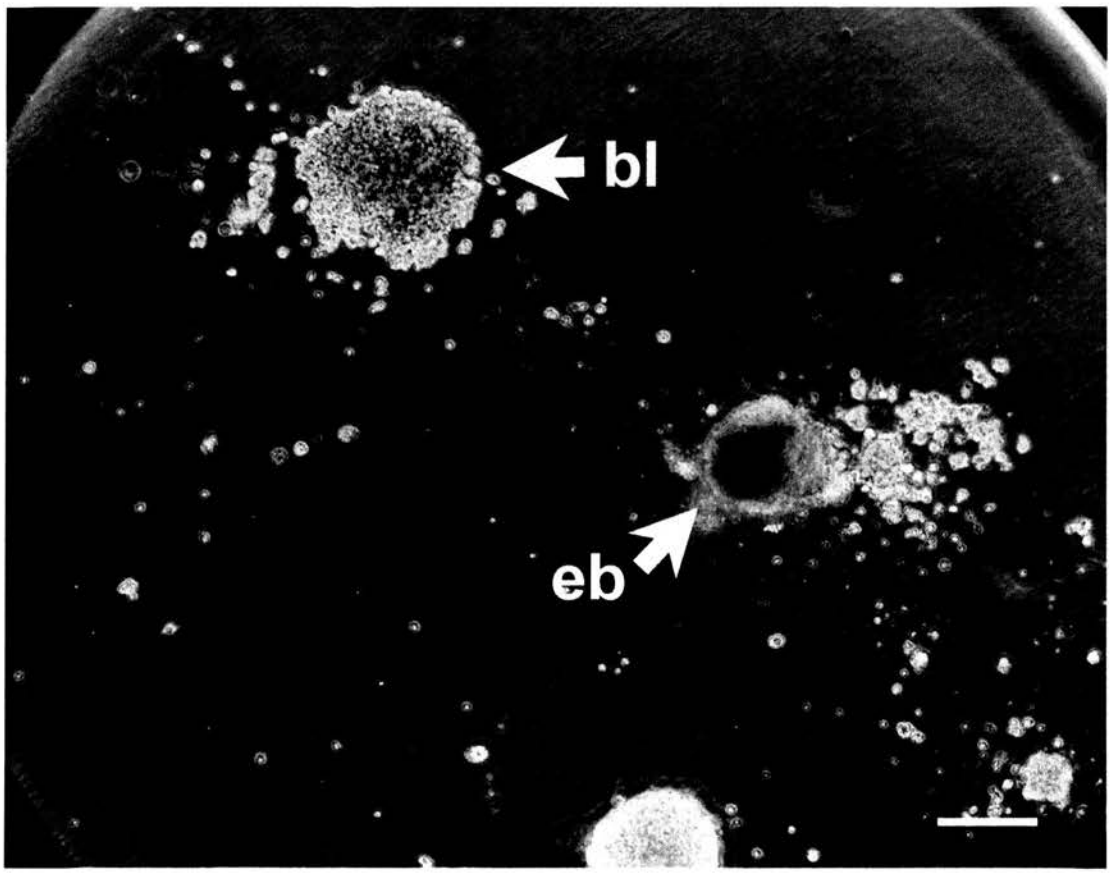

Figure 5.8 *A typical blast colony from a BL-CFC (**bl**) and a secondary EB (**eb**) in a BL-CFC culture assay after 5 days of culture. Scale bar represents 2 mm.*

Keller identified the BL-CFC by morphology alone. Whereas EBs exhibited a tight cohesive morphology and dense packing of small cells, BL-CFCs had a less uniform morphology consisting of larger cells that were less densely packed. The methylcellulose cultures were checked after 4-6 days and all colonies that did not have the dense, compacted and morphologically uniform structure of a secondary EB, was, at this stage, designated a BL-CFC. These colonies were picked and individual colonies were checked for their ability to differentiate divergently to both the haematopoietic and endothelial lineages.

5.1.3.1. Assessment of the Potentiality of the BL-CFC

As following a morphological description of a colony type relies on good observation and accurate qualitative assessment, it was insufficient to simply identify the BL-CFC in this way. To prove the validity of their successful culture, the suggested BL-CFC had to be proved to possess the bipotentiality expected of the *in vitro* equivalent of the haemangioblast.

The blast colonies were picked and each placed in a well of a 12-well plate coated with Matrigel. The cells were grown as described in the Materials and Methods and after 5-6 days of culture, non-adherent and thus, potentially haematopoietic cells were removed and plated in a haematopoietic culture for 7 days to check potentiality for the haematopoietic lineages. To see whether the BL-CFC could differentiate to endothelium, the Matrigel cultures were maintained for an additional 7 days before endothelial markers were detected via immunohistochemistry.

Both primitive EBs and erythroid EBs, as well as more mature CFU-GM and CFU-GEMM were seen in the cultures of non-adherent cells showing that outgrowths from BL-CFCs were able to differentiate down the haematopoietic lineage (see **Figure 5.3** for examples of observed colonies).

Figure 5.9 *Outgrowth of BL-CFCs on Matrigel after 5 days of culture at which time all non-adherent cells (as seen in* ***a****) were removed to haematopoietic cultures. Adherent cells of varying morphology (****b,c****) were cultured on to confluence and stained for markers of differentiation. Scale bar represents 100μm.*

The Matrigel outgrowths of the BL-CFCs were maintained as recommended (Choi *et al.*, 1999) after the removal of the non-adherent cells after 5 days in culture. **Figure 5.9** shows typical wells from the 12 well plates used to grow the BL-CFCs. As can be seen, there were a wide variety of cellular morphologies observed between different outgrowths of the BL-CFCs. **Figure 5.9a** shows the non-adherent cells sitting on the underlying adherent cells before their removal to haematopoietic cultures. A variety of different cell morphologies were seen (**Figure 5.9b and c**) although most were typically endothelial cells with varying growth patterns. The Keller group recommended that the Matrigel cultures be maintained for an additional 1-2 weeks, however it was found that the wells were nearly all confluent after a further 5 days in culture (**Figure 5.10**).

Figure 5.10 *Outgrowths of BL-CFCs grown in Matrigel for 10 days. Although endothelial-like cells were still present in the cultures (****a****), some wells only had cells that resembled muscle cells (****b****). The majority comprised a mix of the two cell morphologies (****c****). Scale bar represents 100μm.*

By this time, the majority of the wells contained a mix of both endothelial, and muscle-like cells (**Figure 5.10c**), although the endothelial cells were outnumbered by

those with muscle morphology (**Figure 5.10 b**). Most cultures also had non-adherent cells that had budded off the adherent cells subsequent to their removal after 5 days in culture. As the wells differed greatly in morphology, the cells were fixed and stained for endothelial and muscle-specific markers to ascertain the cell types present.

As a control, EBs from the BL-CFC cultures had also been plated in Matrigel to see how these differentiated in comparison to BL-CFCs. Although the EBs did grow, no non-adherent cells that looked like haematopoietic cells (uniform circular shape and small size) were seen and the cells growing were disorganised and varied greatly in morphology within cultures of individual EBs (**Figure 5.11**). This would be expected of EBs containing a heterogeneous population of differentiating cells and not a committed progenitor with a determined and already limited cell fate.

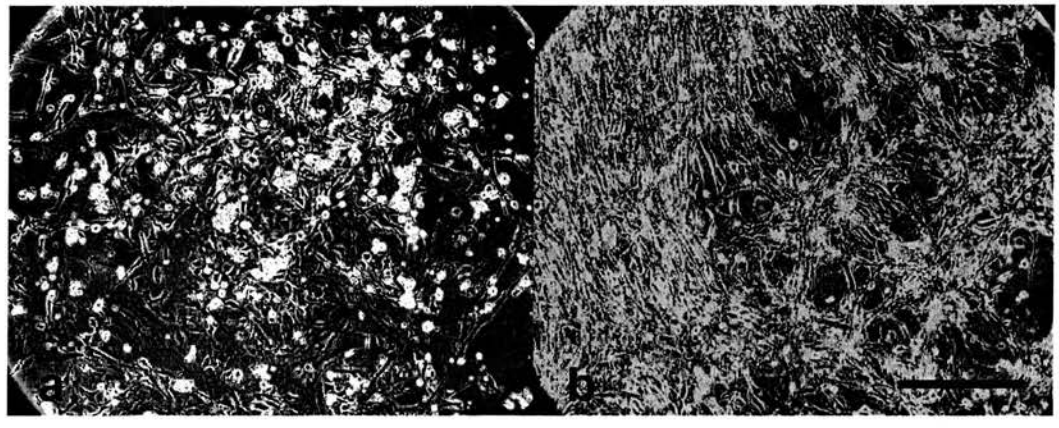

Figure 5.11 *Typical outgrowths of non-blast-like EBs taken from the BL-CFC methylcellulose cultures on Matrigel after 5 (**a**) and 10 (**b**) days of culture. Scale bar represents 100μm.*

5.1.3.2. Immunohistochemical Analysis of Blast Colony and EB Outgrowths

To confirm the haemangioblastic potential of blast colonies, immunohistochemistry using specific markers was used to analyse BL-CFC outgrowths. Outgrowths from secondary EBs were used as controls. Blast colonies derived from BL-CFCs were grown for 5 and 10 days on Matrigel, the cultures were fixed and stained for FLK-1

as well as typical cell surface markers for endothelial (VE-Cadherin), muscle (α-Smooth Muscle Actin (αSMA)) and haematopoietic (SCL/TAL1) lineages.

Where possible, double staining with FLK-1 and one of the other lineage markers was carried out to assess the expression pattern of FLK-1 in the endothelial, haematopoietic and mural lineages.

At each stage when immunohistochemistry was carried out, negative controls i.e. the staining protocol with only the secondary antibodies were included to ensure that any observed staining could not be attributable to non-specific binding of the fluorescently-labelled secondary antibody. See **Appendix E** for examples of negative control samples.

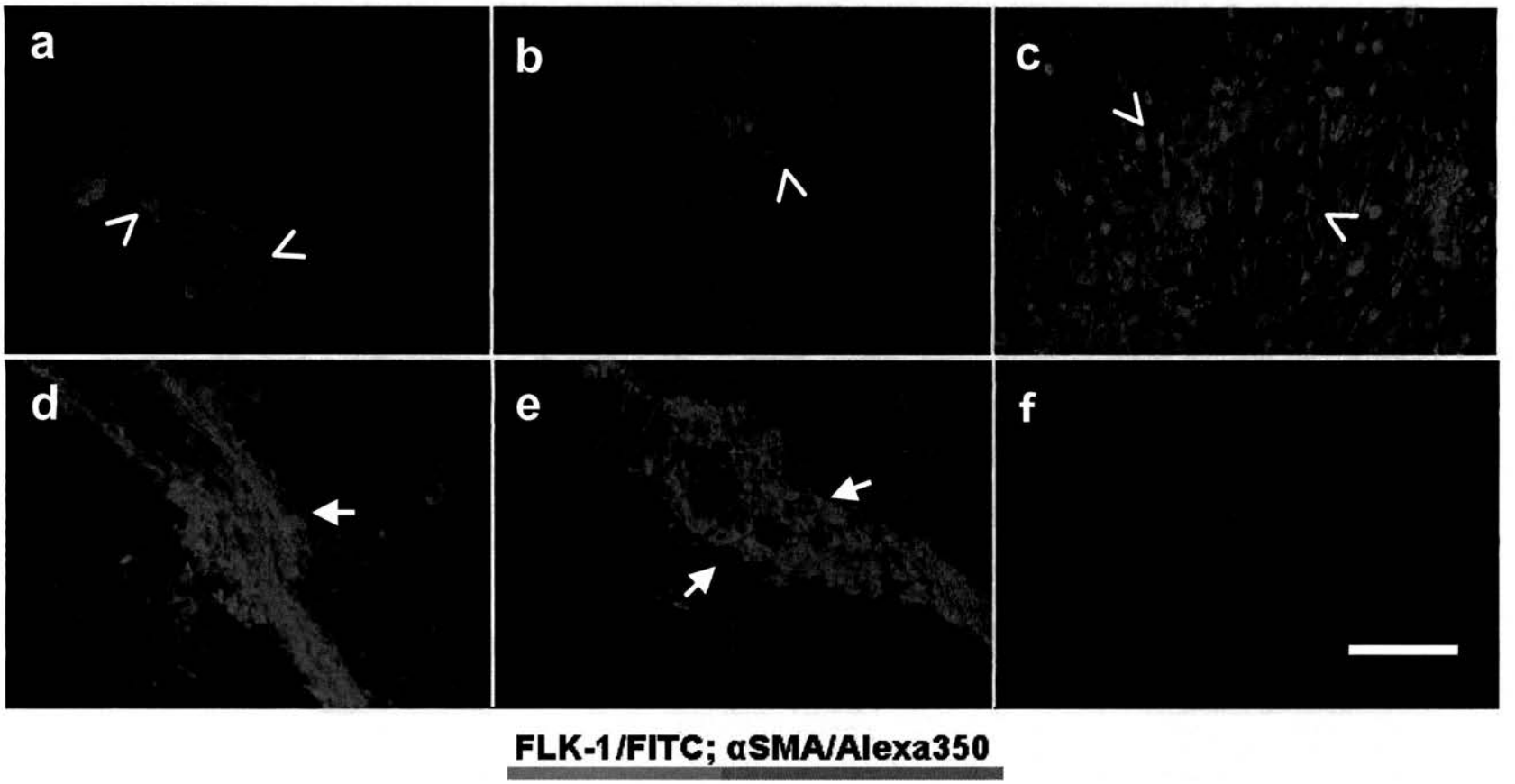

Figure 5.12 *Immunohistochemical detection of FLK-1 and αSMA expression from BL-CFC outgrowths. FLK-1 expression is seen in green, αSMA is light blue (AlexaFluor 350, pale blue) detected in cytoplasm of cells in contrast with the blue DAPI nuclear staining of all cells.* ***a-c*** *show FLK-1 staining alone and the various cell morphologies adopted by FLK-1$^+$ cells.* ***d-f*** *show regions that costain for FLK-1 and αSMA. Arrowheads show regions staining for FLK-1 Arrows show staining for both FLK-1 and αSMA that looked vessel-like in morphology. Scale bar represents 100μm.*

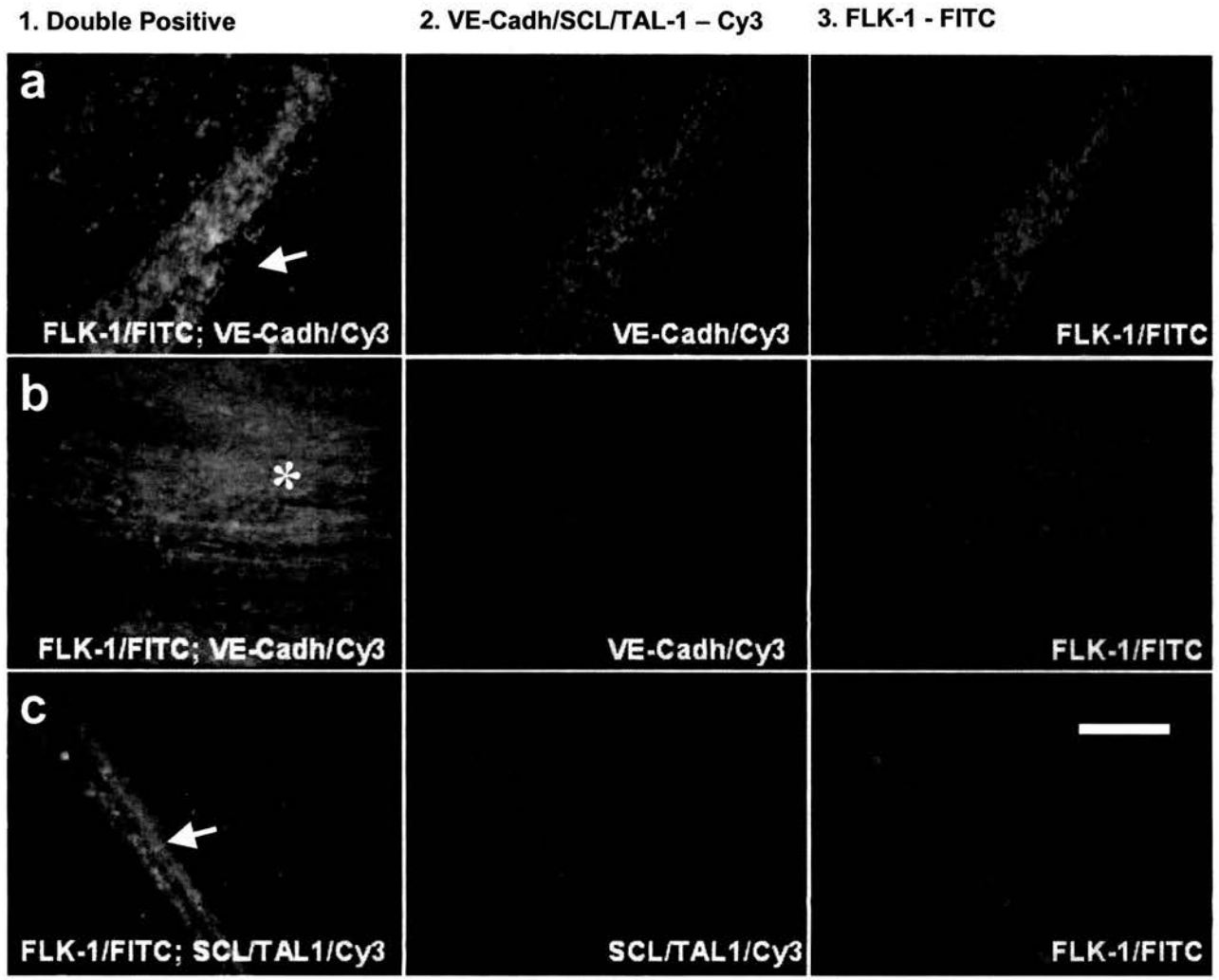

Figure 5.13 *Immunohistochemical staining of blast colony outgrowths after 5 days of culture, for coexpression of FLK-1 with markers of the haematopoietic and endothelial lineages. Double immunostaining with FLK-1 (green) and either VE-Cadherin (**a, b**) or SCL/TAL1 (**c**) (red) antibodies were carried out. Double positive cells appear yellow. DAPI nuclear staining is seen in blue. Vessel-like structures are marked with an arrow. Muscle striations are marked with an asterisk. Scale bar represents 100μm.*

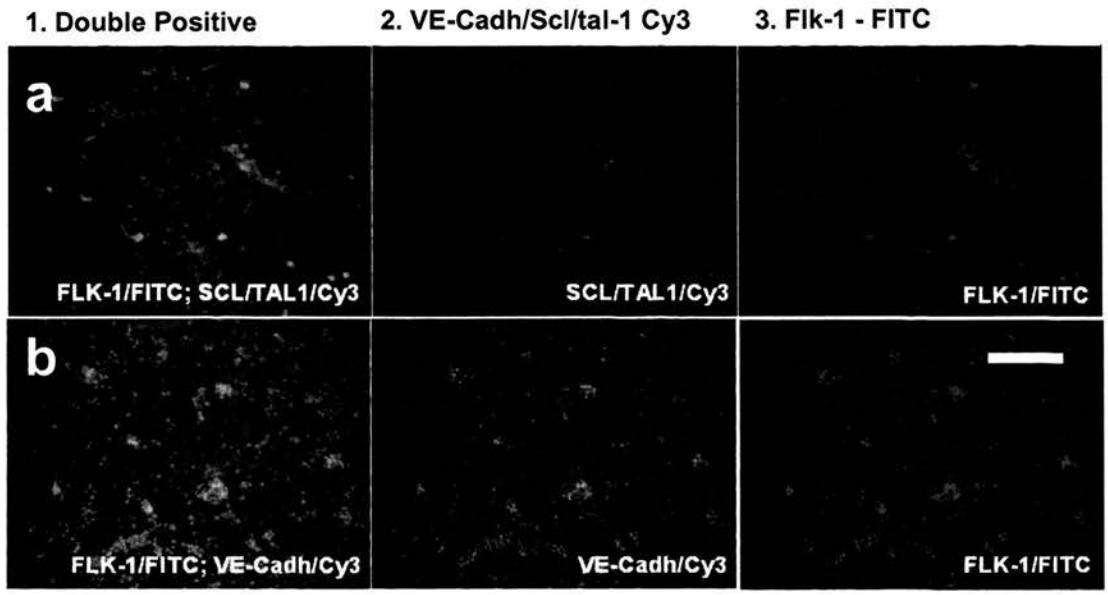

Figure 5.14 *Immunostaining of EBs grown out on Matrigel for 5 days with FLK-1 (green), and either SCL/TAL1 (**a**) or VE-Cadherin (**b**) (red) antibodies. Double positive cells fluoresce yellow. DAPI nuclear stain is seen in blue. Scale bar represents 100μm.*

Figures 5.12 and **5.13** show immunohistochemical staining of outgrowths of blast colonies believed to be derived from BL-CFCs. In **Figure 5.12** FLK-1 staining is carried out with αSMA. The vessel-like structures stain positive for both antibodies. This is most clear in **Figures 5.12d** and **e**. **5.12a** shows an interesting pattern of FLK-1 staining that was more rarely observed and could show the earliest stages of cell elongation and vasculogenesis. **5.12f** shows a striated pattern of FLK-1 and αSMA expression that could represent muscle cell formations.

In **Figure 5.13**, both vessel-like structures (**5.13a** and **c arrows**) and muscle-like fibres (**5.13b asterisk**) are seen. Both differentiated structures stain positively for FLK-1 and VE-Cadherin. The vessels also showed SCL/TAL1 staining although the veracity of this staining was questionable as the antibody staining was not very strong and had not been optimised with a positive control as a suitable cell line was not available. A negative control of undifferentiated cells had been used to eliminate the possibility that the staining seen was non-specific antibody binding (data not shown).

These differentiation experiments were carried out 4 times where single staining for FLK-1 and VE-cadherin were carried out and showed a high level of expression in all cultures. Vessel-like localisation of the FLK-1 antibody was seen as shown in **Figure 5.12a** and **d** and Figure **5.13a** and **c** (approximately 1/20 frequency of observation). However, the most typical pattern of FLK-1 staining was that shown in 5.12c. A pattern of muscle striations was commonly seen (approximately 7/10 frequency of observation) and always corresponded to patchy expression of both FLK-1 and VE-cadherin.

Immunostaining of secondary EBs that had not differentiated at the time of blast colony propagation (4-6 days of growth in methylcellulose) are shown in **Figure 5.14** and in all differentiation experiments, the level of FLK-1 expression was both less broadly seen and where visible, localised to foci. The brightness of antibody binding to these foci was as intense as in the BL-CFC differentiation cultures. αSMA antibody staining was never observed in secondary EB outgrowths however. This may indicate the necessity for BL-CFC to be propagated to drive differentiation through the mural cell fate.

The blast colonies (**Figure 5.12, Figure 5.13**) that were believed to be derived from BL-CFCs displayed a high level of FLK-1 expression and formed vessel-like structures. Although some low level FLK-1 expression was detected in outgrowths from EBs (**Figure 5.14**), no vessel-like structures were seen to form and expression of VE-Cadherin or SCL/TAL1 were weak. Expression of vascular markers from the EB outgrowths was expected, due to the possibilities of random differentiation to the vascular lineages. The lack of vessel-like structures and the comparatively low levels of FLK-1 detection in relation to the blast colony outgrowths were encouraging, and indicated that the BL-CFC culture regime was enabling enrichment for vascular progenitors.

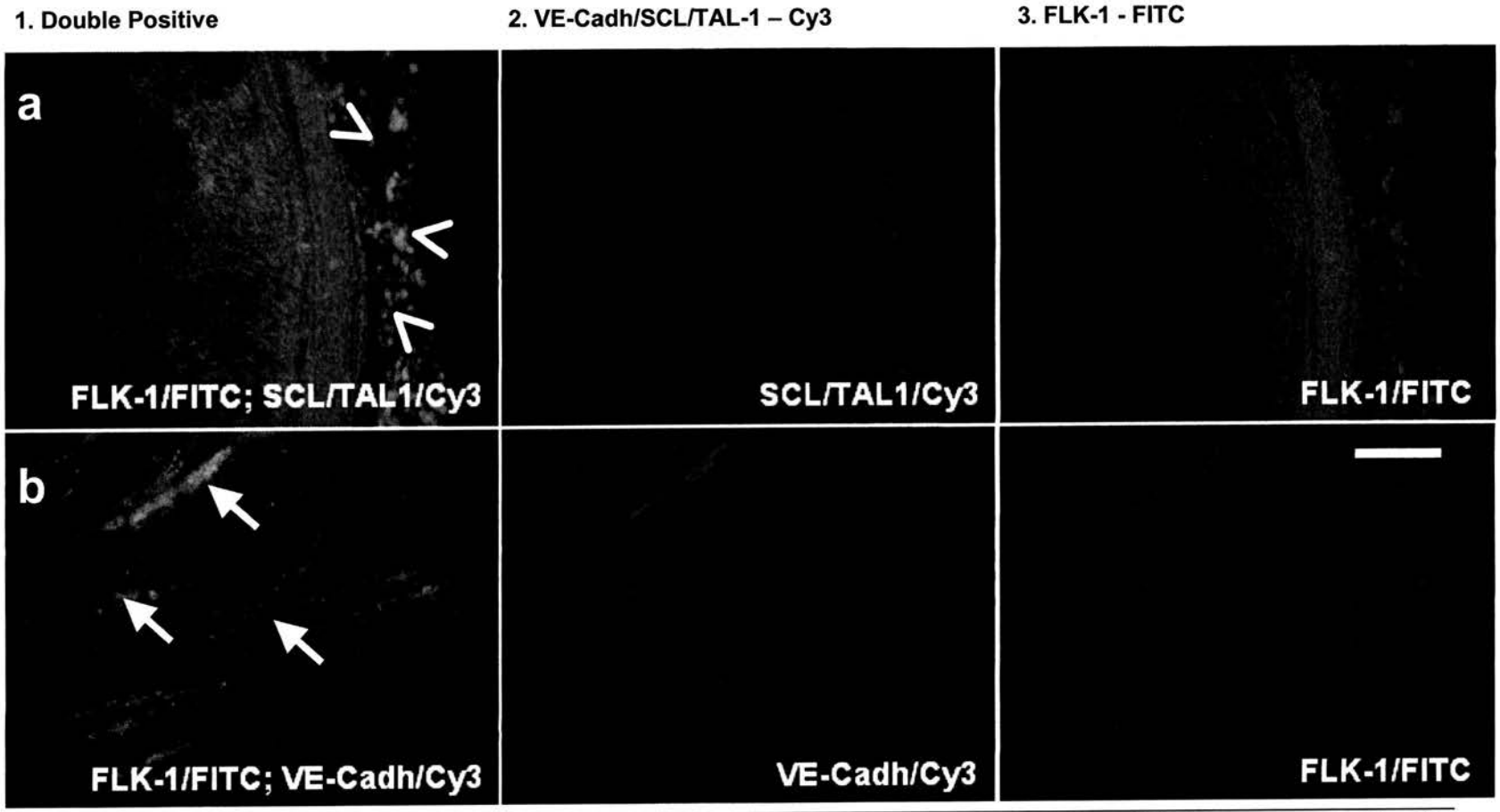

Figure 5.15 *Immunostaining for vascular markers after culture of blast colonies on Matrigel for 10 days: FLK-1 (green), and either VE-Cadherin or SCL/TAL1 (red) Double positive cells fluoresce yellow (**arrowheads**). **Arrows** mark double positive vessel structures. DAPI nuclear stain is seen in blue. Scale bar represents 100μm.*

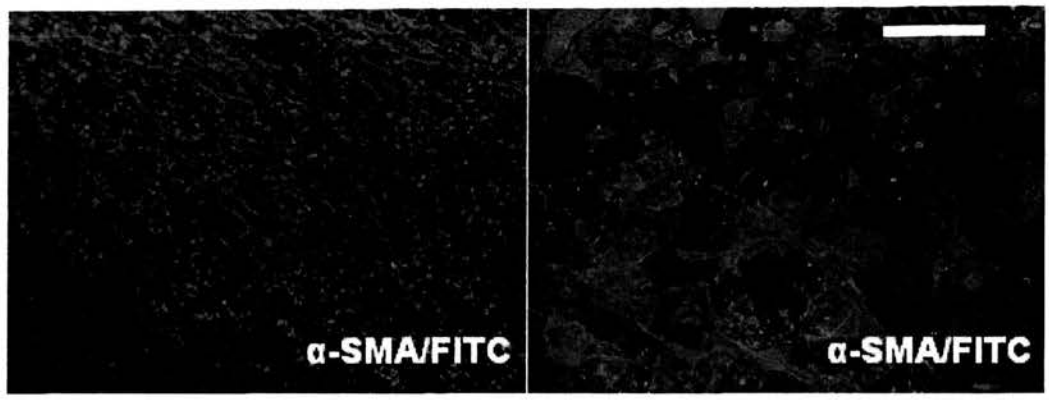

Figure 5.16 *Immunostaining for αSMA (green) in blast colony outgrowths after 10 days. DAPI nuclear stain is seen in blue. Scale bar represents 100μm.*

10-day-old outgrowths of the blast colonies were reassessed for FLK-1 expression alongside endothelial, haematopoietic and mural markers. Although at this stage many of the wells were very confluent and displayed muscle-like cellular morphologies, staining revealed vessel-like structures amongst the dense cell mass that co-stained for the endothelial VE-Cadherin marker and slightly for the haematopoietic SCL/TAL1 cell marker as shown in **Figure 5.15**. There was a considerable level of muscle formation too however, with two distinct morphologies seen (**Figure 5.16**) depending on the confluence of the wells at the time of staining. Staining for these vascular markers was carried out twice and in both instances these vascular structures were observed amongst the αSMA staining cells that dominated the cultures.

5.2. Discussion and Conclusions

5.2.1. Optimisation of the BL-CFC Culture Assay

The BL-CFC assay was not straightforward and needed optimisation before it was carried out successfully. The variables that were considered to be important in the success of the assay included the age of EBs introduced into the assay, the disaggregation method, the quality of the D4T conditioned medium and the concentration of the growth factors added into the assay. Using CCE ES cells (Robertson *et al.*, 1986), it had been shown that BL-CFC existed in EBs from 2.5 days to 3.5 days of differentiation with maximal blast colony producing potential observed at 2.75 days of differentiation (Kennedy *et al.*, 1997). However, as a different ES cell line was being used for these studies, this timescale could have differed for the HM1 ES cell line and its *flk-1* targeted derivative cell lines. Although the earlier stages in the development of the BL-CFC culture regime were carried out in the absence of D4T conditioned medium, indicating that its addition is not the critical step in the success of the colony assay, if the D4T cells were secreting factors that mitigated the potential for cells to grow in methylcellulose, they could affect the rate of success of the colony assay. Also, as the assay was a very specific one, the absence of the D4T CM, coupled with the HM1-derived ES cell line's possible poorer plating efficiency in methylcellulose or a different timescale of differentiation would have affected the plating efficiency required for the success of the assay.

5.2.1.1. The Importance of the Method of EB Disaggregation on Successful Methylcellulose Differentiation

As elaborated on in the results section, basic haematopoietic differentiation assays were carried out in order to assess the importance of disaggregation methods and to subsequently introduce elements of the BL-CFC culture protocol to the methylcellulose to assess their influence on the success of the assay.

Figures 5.4 and **5.6** show the results of two experiments comparing trypsin and dispase disaggregation methods. Both experiments showed that the dispase disaggregation method had a strong impact on the viability on the cells and, as a consequence, the number of colonies yielded in the haematopoietic assay. As different time points (2.75, 3, 3.5 and 4 days of differentiation) were taken in the setup of the experiment, it was evident that with HM1 ES cells, the 2.75-day timepointwas the best for BL-CFC differentiation in accordance with observations made by Kennedy *et al.*, (1997).

Earlier timepoints should have been analysed if it was to be shown that 2.75 days of differentiation was optimal. However, EBs at that stage of differentiation are very small and it was seen to be sufficient to use the best timepoint out of those tested. Kennedy *et al.* had observed a 24-hour window of BL-CFC presence in EBs. It appeared that the HM1 ES cells at least had a higher proliferative potential at the earliest stages of differentiation. It was therefore decided to use the 2.75 day timepoint from that point forth, at least until all other parameters for BL-CFC had been optimised. The other parameters had to be addressed first so that a yield of blast colonies could be obtained (rather than colony-forming or haematopoietic potential as was the case at this stage).

Another observation was that the number of secondary EBs dropped and more mature haematopoietic colonies arose as the time in the differentiation cultures increased. This indicated that the colonies forming were healthy and had the potential to develop beyond secondary EBs, which only show the proliferative potential of the cells plated in the methylcellulose, to form differentiated haematopoietic colonies as seen in **Figure 5.3**. This trend was summarised in **Figure 5.5** and **5.6**. The latter showed the direct increase in haematopoietic colonies as time of differentiation increased and the number of secondary EBs dropped.

5.2.1.2. Conditioning Regime had Little Influence on Plating Efficiency

By comparing the colony numbers after addition of conditioned medium to the haematopoietic assays in **Figure 5.7** with the colony numbers seen in the previous experiments, it was evident that the conditioned medium enhanced the plating efficiency of the cells in the assays resulting in a higher frequency of secondary EBs being observed after 7 days of culture. The length of time allowed for conditioning or the presence of ECGS of the conditioning regime did not appear to significantly affect the efficiency of the culture method. A control that should have been incorporated into this experiment was the culture of cells in the absence of any conditioned medium. This would have been a useful measure of the importance of the conditioned medium in the assay as well as the level of assay variability that could be attributed to the conditioned medium.

Since these experiments were carried out, Gordon Keller and Marion Kennedy have published a detailed description of how they carry out the BL-CFC culture, including the production of the D4T conditioned medium (Kennedy and Keller, 2003). Their method also involves the use of medium without ECGS and is carried out for 3 days. They used the D4T cells at confluence and repeated the conditioning up to 5 times. However, they did suggest that the conditioned medium should be tested each time it is prepared, for its ability to support the growth of blast colonies in the presence of VEGF and IL-6 in order to standardise the culture regime (Kennedy and Keller, 2003).

5.2.1.3. Progression to a Complete BL-CFC Assay

The only step that remained following the assurance that the D4T conditioned medium was not negatively affecting the success of the BL-CFC cultures was to remove the haematopoiesis-inducing growth supplements and replace them with the KL and VEGF growth factors to complete the BL-CFC assay and see whether blast colonies capable of the multipotentiality of the haemangioblast could be isolated.

The transition to carrying out the full BL-CFC culture was successfully completed on the first attempt and yielded many colonies. The blast colonies were distinguished from the secondary EBs using descriptions and published photographs (Kennedy *et al.*, 1997; Choi *et al.*, 1998; Faloon *et al.*, 2000; Robertson *et al.*, 2000; Kennedy and Keller, 2003). However, although the difference between secondary EBs and blast colonies was easy to distinguish, the appearance of such colonies was not limited to the BL-CFC assay and were often seen in previous haematopoietic colony assays before the colonies ruptured to form the various haematopoietic colonies seen. It would have been interesting to see whether these blast colonies behaved similarly if they were not picked into Matrigel but were maintained in KL and VEGF for a longer culture duration. Would they have differentiated to a variety of haematopoietic lineages or would they have required prompt stimulation down a haemangioblastic lineage to prevent cell death?

5.2.2. Potentiality of BL-CFCs

Both blast colonies and secondary EBs were picked and cultured on Matrigel to assess the colonies for their potential to differentiate down the haematopoietic and endothelial lineages. The nonadherent cells were separated from these cultures and when grown in methylcellulose-based haematopoietic assays, a range of erythroid and myeloid haematopoietic colonies were observed (see **Figure 5.3** for examples). Ideally, all three haematopoietic lineages (myeloid, lymphoid and erythroid) should have been identified to prove the haematopoietic potentiality of the blast colonies. The remaining adherent cells were cultured for a further 5 days on Matrigel. Most of the colonies displayed a similar course of differentiation. They adhered and spread when plated out and after initially exhibiting endothelial-like morphologies, the dense cells formed muscle-like fibres and swirls. Staining revealed dense vascular-like networks that could not be identified by the naked eye (**Figures 5.13** and **5.15**). These putative vessels showed strong FLK-1 and VE-Cadherin expression indicative of endothelial cells. Although some staining for SCL/TAL1 was seen, confidence in the efficacy of the SCL/TAL1 antibody was low and what staining was seen was very patchy. If these observations are real, patchy and localised SCL/TAL1

expression along the vessel walls could be understood to support the argument for the haemogenic potential of vessel endothelium (**Chapter 1, 1.10.6**).

5.2.3. Differentiation of the BL-CFC to αSMA$^+$ Muscle Cell Fate

The research carried out on the BL-CFC to date has shown its ability to differentiate to form cells from the haematopoietic and endothelial lineages. The BL-CFC cultures carried out in the present body of work confirm this and also demonstrate that the blast colonies, supposedly derived from BL-CFCs, show a strong differentiation capability to form αSMA expressing smooth muscle cells (**Figure 5.16**). This agrees with the findings of Yamashita *et al.* (2000) who found from their analysis of haemogenic endothelial cells differentiated on collagen IV that the cells also had the ability to differentiate to form smooth muscle cells.

5.2.4. Are BL-CFCs *In Vitro*-Derived Haemangioblasts?

The secondary EBs taken from the BL-CFC cultures did proliferate after plating on Matrigel. However, in general, their growth was slower and phenotypically, the range of cell morphologies seen was far more restricted than that observed from outgrowths of blast colonies (data not shown). Also, Matrigel cultures of EBs yielded fewer confluent wells of cells (data not shown). Although staining for FLK-1 was seen in the Matrigel cultures of the EBs (**Figure 5.14**), no αSMA staining was seen and the SCL/TAL1 and VE-Cadherin staining was questionable. Certainly no morphologically distinct vascular structures as seen in the blast colony cultures were seen.

It may have been the case that the EBs were able to show some response to stimulation by KL[1] and VEGF ligands but that they were not primed to coordinate an organised response to these ligands as a result of the absence of VEGF and the c-Kit receptors. This coordinated response together with growth on a permissive adherence substrate, in this case Matrigel, probably provided sufficient stimulation to drive the blast colonies, which, when picked from methylcellulose, lack a cohesive structure or distinct cell morphologies, to form blood vessels and differentiate to all the lineages seen in blood vessels. This does indicate a difference between the picked blast colonies and EBs with respect to differentiation potential. However, to confirm that these blast colonies were derived from haemangioblastic BL-CFCs, molecular analysis of the expression patterns of the two types of colonies would need to be carried out and tracked throughout differentiation under different stimuli.

Gordon Keller's group have convincingly shown that a cell type can be isolated from EBs between 2.5 and 3.5 days of differentiation. This so-called BL-CFC can be selected for using the blast colony culture regime and can be shown to have potentiality to the endothelial and haematopoietic lineages. In the above experiments it was also seen to differentiate into muscle-like fibres in accordance with studies using the Collagen IV haemangioblast differentiation protocol (Yamashita *et al.*, 2000) that found that their bipotential cells were also able to differentiate to αSMA expressing muscle.

What has not been shown of the BL-CFC is whether its potentiality is in fact limited to the haemangioblastic lineages. When the EBs are disaggregated and cells plated into the BL-CFC cultures in methylcellulose, the growth factors are believed to only stimulate the growth of colonies from haemangioblastic BL-CFC from the otherwise heterogeneous cell population. If this BL-CFC could be isolated (prior to its differentiation and induction of blast colony formation) using its molecular profile, it

[1] The exact role of c-kit and its ligand in haematopoiesis are unclear. They are expressed on YS-derived precursors and stem cells found in the foetal liver (Ogawa *et al.*, 1993; Ikuta and Weissman, 1992). What is known is that they are not directly required for the establishment of the haematopoietic system (Kabrun *et al.*, 1997).

would be interesting to see whether it could respond to stimuli that could direct it to other lineages or whether it truly is limited to the haemangioblastic cell fates.

Both the Keller and Nishikawa (discussed in the following chapter) differentiation protocols were devised using a different ES cell line (CCE ES cells in both protocols (Robertson *et al.*, 1986; Nishikawa *et al.*, 1998a)) and different growth conditions to those used in the isolation and maintenance of the HM1 cell line. ES cells are known to adapt to their isolation and growth conditions. Not only that, but the varying characteristics and responses observed in different ES cell lines under pluripotential as well as differentiating conditions mean that any differentiation protocol devised for one ES cell line is possibly going to have a different effect on another cell line. At the very least, it may be less effective in generating the desired cell type or lineage. The difficulties encountered in the Keller differentiation protocol may be attributable to the use of a different ES cell line for the differentiation experiments. The fact that CCE ES cells appear to be the ES cell line of choice for many vascular and haematopoietic differentiation investigations may indicate that a characteristic of the line is easy induction of these lineages.

This highlights an inconsistency and a major disadvantage of *in vitro* modelling of *in vivo* events. Each ES cell line is affected by its means of isolation and subsequent culture. Although crucially, each line is ultimately pluripotent, subtle variation between lines with respect to growth, ease of differentiation to particular pathways and vigour can have more profound influences on complex manipulation of the ES cell lines and the observations and conclusions drawn from such investigations. Ideally, several ES cell lines should be tested in a differentiation protocol as a measure of the robustness of the protocol and the results obtained from it.

5.2.5. Use of the BL-CFC Assay to Isolate Pure FLK-1$^+$ Cell Populations

Following successful repetition of the BL-CFC assay, attempts were made to incorporate HAT selection with the differentiation culture in conjunction with the use

of *flk-1*/HPRT targeted ES cells. Despite many attempts, HAT could not be optimised with the BL-CFC culture technique irrespective of the time at which HAT was added. In light of the technical difficulties that prevented the isolation of a pure population of FLK-1$^+$ cells with the BL-CFC assay, the Nishikawa protocol for the isolation of FLK-1$^+$ cells differentiated on collagen IV was optimised for FLK-1$^+$ cell yield first, then for the isolation of the derived FLK-1$^+$ cells following the addition of HAT to the cultures (see Chapter 6).

5.3. Summary

- The BL-CFC culture regime proved to be highly problematic and required much practise and optimisation.

- Although finally carried out successfully, the putative haemangioblastic cells were never assessed for their *in vivo* repopulation ability.

- The BL-CFC culture system was found not to be compatible with a FLK-1 selection regime due to the variability and inherent sensitivity of the culture technique.

CHAPTER 6

Directed Differentiation for Flk-1 Expression II: Nishikawa Protocol

Directed Differentiation for Flk-1 Expression II: Nishikawa Protocol

6.1. Introduction

6.1.1. Two-Dimensional Differentiation on Collagen IV

The need to form the EB structure has been shown to be dispensable following the differentiation of ES cells on feeder cells (Gutierrez-Ramos and Palacios, 1992; Nakano *et al.*, 1994) although it was not proved whether defined haematopoietic differentiation was independent of three-dimensional cell morphologies that may have formed during the differentiation procedure. Nishikawa and colleagues set out to determine whether it was also possible to attain haematopoietic and vascular differentiation in a two-dimensional culture system.

This alternative method of ES cell differentiation for the haematopoietic and vascular lineages involves the growth of ES cells seeded at very low density on collagen IV, which is the principal constituent of the basement membrane (**Figure 6.1**). The basement membrane supports overlying epithelial or endothelial cells and functions as a selective barrier for macromolecules. It consists of the basal lamina and an underlying network of reticular collagen. When first investigating substrates that would support mesodermal and haematopoietic differentiation without an EB generation step, gelatin, fibronectin, type I and type IV collagens were tried with collagen IV being found to be the most effective (Nishikawa *et al.*, 1998a). Also, it was discovered that differentiation of VE Cadherin$^+$ endothelial cells was enhanced by the addition of SCF and VEGF, and that it was not required for FLK-1 induction or differentiation of cells of the vascular system.

Utilising this culture regime coupled with FACS sorting and analysis of the various distinct populations of cells that arose from FLK-1$^+$ cells, Nishikawa and colleagues examined the segregation of the FLK-1 expressing cell population differentiating on collagen IV, the kinetics of differentiation and the expression profile and subsequent cell fate of the different cells that arose from the early FLK-1$^+$ sorted population.

They used this *in vitro* experimentation system to try and replicate the sequence of events that take place in the developing embryo and the potentiality of cells bearing specific marker profiles (Nishikawa *et al.*, 1998a; Nishikawa *et al.*, 1998b, Hirashima *et al.*, 1999; Hirashima *et al.*, 2003). They isolated FLK-1$^+$ cells from a heterogeneous population; this in itself increased the appearance of blood progenitors. Using FACS they followed the expression profile of these cells under different growth conditions as they differentiated.

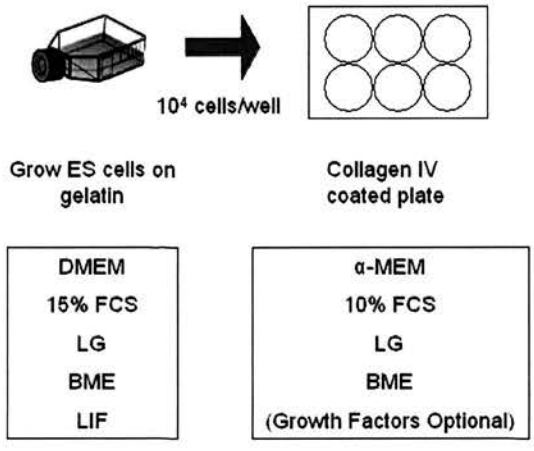

Figure 6.1 *Diagram showing the steps involved in the two-dimensional differentiation of ES cells on collagen IV to express FLK-1, as devised by Nishikawa et al. (1998). LG, L-glutamine; BME, β-mercaptoethanol; SCF and VEGF growth factors could also be added to increase the yield of VE-Cadherin$^+$ cells.*

6.2. Aims

- To successfully replicate and optimise the Nishikawa collagen IV differentiation regime with the *flk-1*/HPRT targeted ES cell lines.
- To optimise differentiation protocols for the expression of FLK-1.
- To devise a selection regime to isolate a pure population of FLK-1 expressing cells for *in vivo* transplantation.

From preliminary differentiation and HAT selection trials described in Chapter 4, it was apparent that the HPRT transgene was functional and that the HAT selection regime seemed to work. However, the protocol for differentiation and application of the selection regime needed to be optimised for the highest level of enrichment for

FLK-1 expressing cells. Ideally, the FLK-1$^+$ cells isolated should be progenitor cells and not terminally differentiated endothelial cells so that after *in vivo* transplantation, their potentiality is greater, in turn broadening the potential for their use therapeutically.

6.3. Optimisation of a Selection Regime for *Flk-1*/HPRT Expressing Cells

6.3.1. Experimental Outline

Preliminary attempts using the collagen IV differentiation protocol to deduce the functionality of the HPRT transgene in Chapter 4 were repeated with the HAT selection applied at three initial timepoints of 0, 2 and 4 days after plating on collagen IV. The plates were observed daily to see whether cells were surviving the selection and proliferating as would be expected of *flk-1*/HPRT expressing progenitor cells. Nishikawa *et al.,* (1998a) reported the presence of *flk-1* expressing cells as soon as one day after plating at low density (10^4 cells/well of a 6 well plate) on collagen IV coated plates. However, in the current experiments, the ES cells plated out on collagen IV did not display any signs of growth until approximately 48 hours post-plating. Although there was variation between different experiments, sometime after 48 hours post-plating there was a noticeable increase in cell number on the collagen IV plates. The differentiating ES cells both proliferated and spread across the surface area of the plates. The decision was taken to apply HAT selection at 0, 2 and 4 days post-plating, based on this observation and under the assumption that the population explosion and differentiation may have been correlated with the expression of *flk-1* and the differentiation of the ES cells to the vascular lineages. The expectation was that 2-4 days post-plating would be the optimal time point for maximal yield of *flk-1* progenitors that had yet to terminally differentiate to the endothelial lineage. The same procedure was repeated with the HM1 parental ES cell line as a negative control. As these cells do not possess the *hprt* gene, they were expected to die in HAT selection and give an indication of the speed of the selection regime before assessment of the number of HPRT$^+$ cells in the experiment using *flk-1*/HPRT targeted cells.

6.3.2. Results

Figure 6.2 shows the results of one of three such trials, all of which were similar in trend. Each growth condition or treatment within each experiment was carried out in triplicate to guard against spurious observations. The cells placed directly into selection on the day of plating on collagen IV did not plate down at all (**Figure 6.2 b** shows the cells 2 days after differentiation and selection were applied). This is unsurprising given that the cells would not have had time to respond to the inductive influence of the collagen IV substrate before selection for FLK-1 expression took effect. Cells that were allowed to differentiate for 2 days before selection was applied showed poor and short-lived cell survival after selection for two subsequent days. Although they had adhered and formed dense colonies on the collagen surface, there had been little proliferation before selection was applied (**Figure 6.2 c, d**). The cell survival from all the selection plates was poor although more viable and endothelial-like cells were propagated from the wells that were placed under selection after 4 days of culture on collagen IV (**Figure 6.2 f, h**). The control HM1 cells showed the fastest level of death, however (**Figure 6.2 i, j**), indicating that a survival advantage was conferred by the HPRT transgene as hoped for. This suggested that the HAT selection should be applied at a later stage and that *flk-1* expression in these ES cells was induced at a later stage than observed by the Nishikawa group. ES cells plated at 10^4 cells per well of a 6-well plate reached confluence by the eighth day of differentiation. To try and capture the cells in a highly proliferative state, selection was thought to be better if applied before cell confluence was reached. The experiment was therefore repeated with HAT selection applied after the sixth day of differentiation.

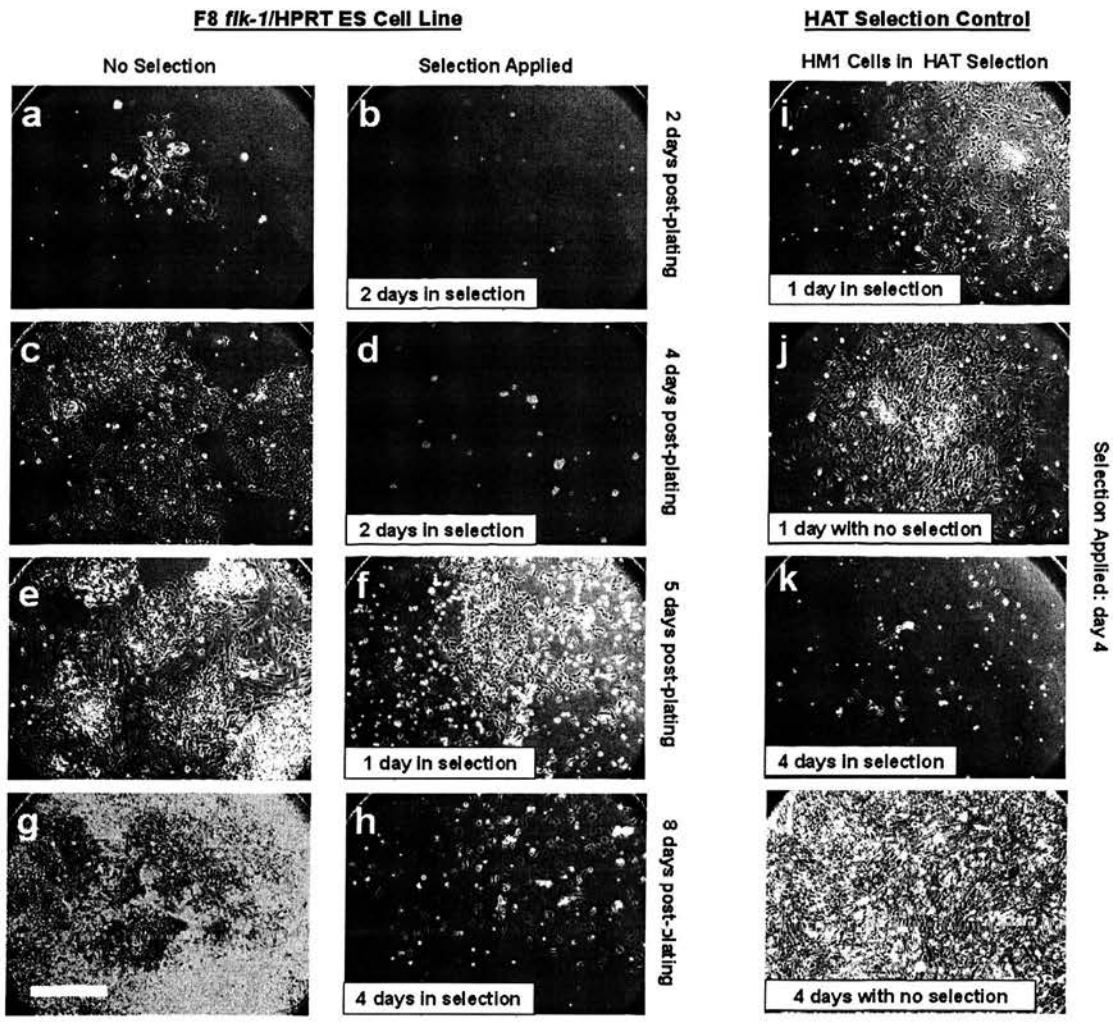

Figure 6.2 *Cell number and morphology of flk-1/hprt ES cells growing with (**b, d, f, h**) and without (**a, c, e, g**) HAT selection. HAT selection was applied at various timepoints: at the time of plating on collagen IV: day 0 (**b**), and at 2 (**d**) and 4 days (**f, h**) after plating. Cultures were maintained until 8 days after plating when the control plates were fully confluent (**g**). HM1 ES cells were used as a negative control to assess the level of cell death expected from a completely HPRT deficient ES cell line (**i,kj**). Cells maintained under identical growth conditions in the absence of any selection are shown in **j** and **l**. Scale bar represents 200μm. See Appendix C for further pictures.*

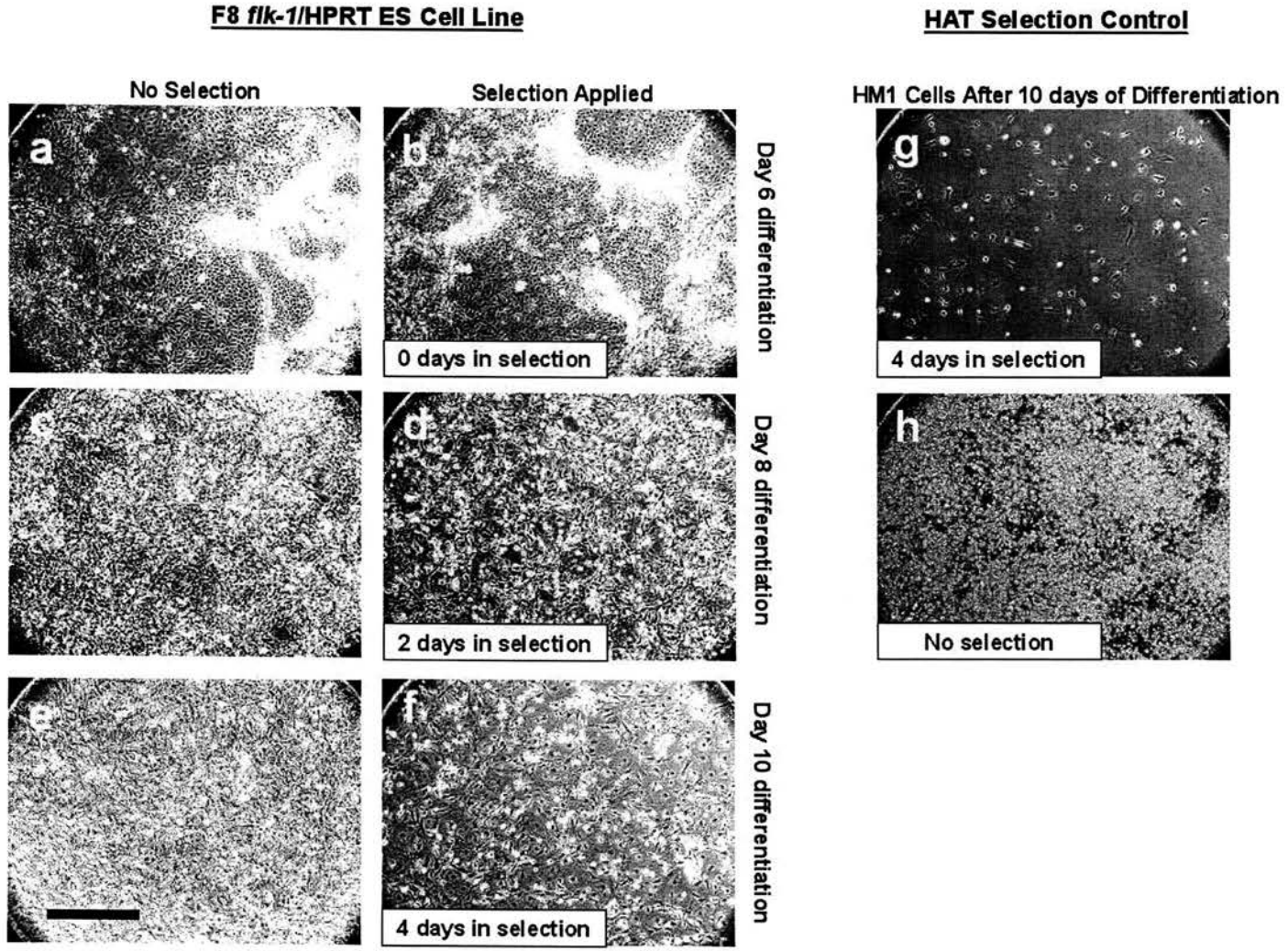

Figure 6.3 *Course of selection and cell death after HAT selection is applied at 6 days of differentiation of flk-1/HPRT ES cells on collagen IV. Cells in and out of selection are shown at 6 (**a, b**), 8 (**c, d**) and 10 (**e, f**) days of differentiation. HAT sensitive HM1 ES cells that have been placed in selection for 4 days are shown (**g**) alongside those that have not been placed under HAT selection (**h**). Scale bar represents 200μm. See Appendix D for further pictures*

When applied after 6 days of differentiation, selection for a proliferating population of endothelial-like cells was far more effective than when applied at earlier stages (**Figure 6.3**). Cell death was apparent after 24 hours, and by 48 hours a clear morphological distinction could be made between cells in the presence (**Figure 6.3 d**) and absence (**Figure 6.3 c**) of selection. The selected cells were endothelial in morphology: displaying typical "cobblestone" contours and uniform size. In the absence of selection, cells formed a densely packed monolayer where cell morphology could not be easily discerned and the cells on the surface were highly refractile. HM1 ES cells were placed under selection in parallel with the *flk-1*/HPRT ES cell lines as a control for cell death. All HM1 cells should die in HAT selection and were used as a guide to assess how long it took for all non-expressers of FLK-1 and the HPRT transgene to be killed. The HM1 wells were clearly sparser than the others, however, even after 4 days of selection a few cells still remained attached to the wells.

As the HM1 line is HPRT deficient, it was expected that all cells would be killed off effectively on application of HAT selection. However, observing these cells "surviving" despite several days of selection, led to the mode of action of HAT being considered. HAT has a selective influence on actively cycling cells in which there would be a need for purine synthesis and therefore a reliance on the activity of HPRT in the presence of the aminopterin inhibitor. If the differentiating cells had senesced and stopped cycling before the HAT selection had taken effect, they would not be killed by its presence. This had implications for the purification of a pure *flk-1* expressing progenitor population. Cells in the differentiating *flk-1*/HPRT ES cell populations could senesce and lose the ability to respond to HAT selection. If the level of cell survival seen in the HM1 population was an accurate reflection of the level of contamination from senescent cells, then this was not an issue, however, it cannot be assumed that the level of contamination was as low each time. An added consideration was the possibility of there being terminally differentiated and quiescent endothelial cells that had exited the cell cycle and were therefore also resistant to the effects of HAT selection. The collagen IV plates were found to be variable between batches so it was never taken for granted that an observation would be repeatable with a different batch of plates.

6.3.2.1. Elimination of Non-Cycling Cells

In order to eliminate any non-cycling cells that were resistant to HAT selection, a trypsinisation and replating regime was inserted into the selection protocol. **Figure 6.3** shows the path of differentiation and selection after optimisation from the point of plating 10^4 cells per well of a collagen IV 6-well plate. A noticeable level of cell death was visible after 24 hours of HAT selection in comparison with cells not in selection; the selection regime was consistently seen to take effect quickly. 24 hours after selection was introduced, the cells were trypsinised and replated in a new well. Replating the cells after a longer period of selection resulted in a lower plating efficiency and a loss of proliferation in the cell population in comparison with cells in selection for the same length of time but which had undergone the replating step at an earlier stage of selection. The best selection regime established, (**Figure 6.4 A**) involved a 24-hour selection period before replating and subsequent maintenance of the cells in selection. The number of cells dying in selection was at its peak after 24 hours of selection. In control plates of wildtype cells, residual cells did survive the selection procedure, presumably due to the fact that they were not metabolically active and therefore insensitive to the HPRT selection method employed. After replating, the *flk-1*/HPRT cells that attached all had a uniform endothelial-like morphology. Cell death continued at a lower but steady level thereafter until after about 2 weeks of differentiation, few cells remained. Those that did had a high cytoplasm to nucleus ratio and no longer had a tight uniform morphology. **Figure 6.4 B** shows HM1 ES cells placed in selection in parallel with the F8 clone of the *flk-1*/HPRT ES cells. HM1 cells were successfully eliminated following the replating step.

Adding a disaggregation and replating step to the collagen IV differentiation protocol and selection regime aided the isolation of a morphologically uniform endothelial-like *flk-1*/HPRT expressing cell population. These cells could be maintained in selection for over a week, however, little proliferation was observed and the cells could not be expanded. The possible reasons for the failure to grow the selected cells for a longer period of time are discussed in the discussion section of this chapter.

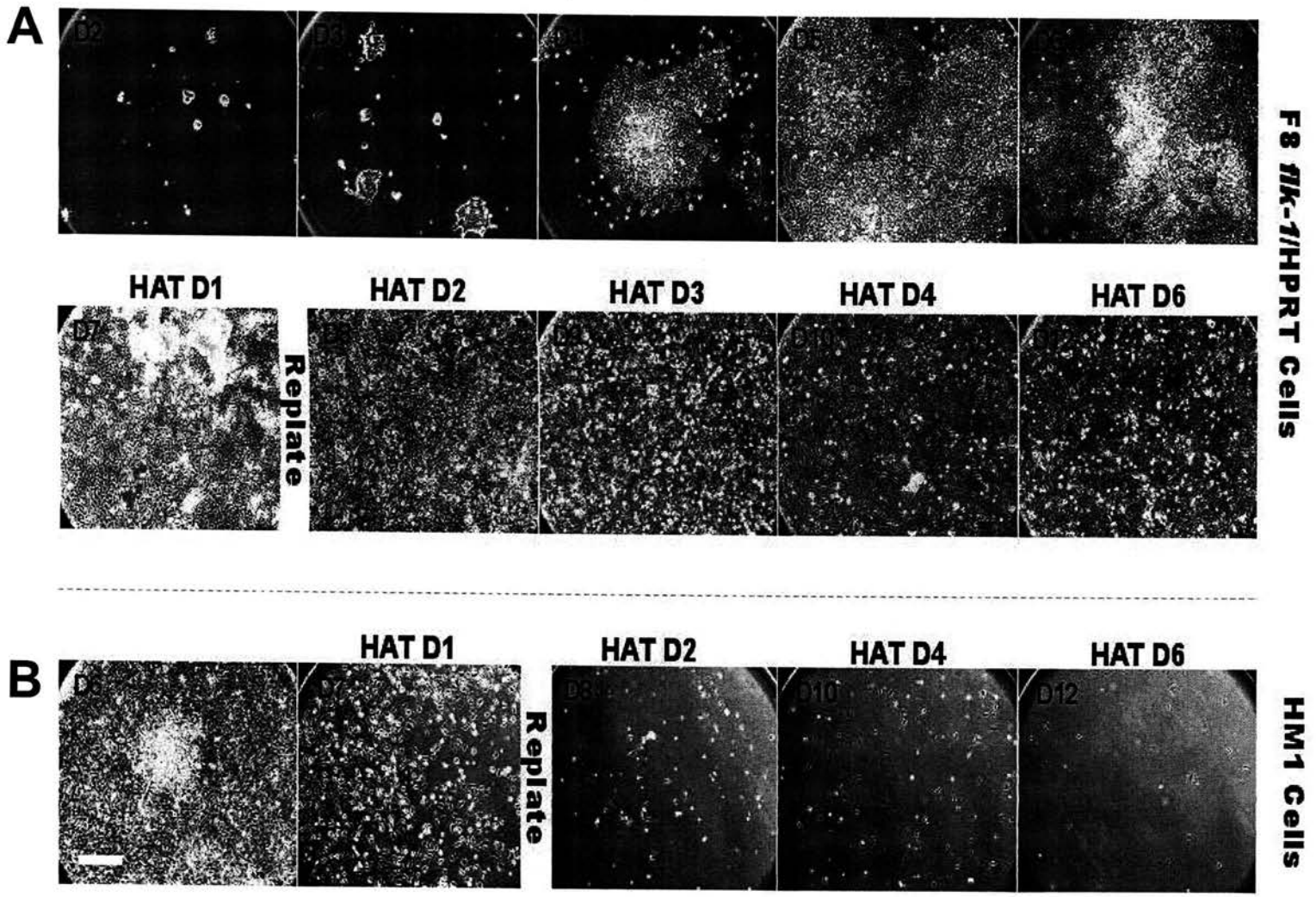

Figure 6.4

A *Morphology and cell density of differentiating cells grown on collagen IV using the optimised protocol for differentiation and replating of the cells shown between days 2 and 12 after differentiation was initiated.*

B *HAT sensitive HM1 parental ES cell line, under the same differentiation and selection regime.* ***D2-D12*** *indicate the day after differentiation was initiated that the cells were photographed. Scale bar represents 200μm.*

6.3.3. Transcriptional Profile of Differentiating Cells With and Without of Selection

In order to determine whether the FLK-1$^+$ cells were progenitors or mature endothelial cells, RT-PCR was carried out on isolated RNA from cells at different stages of differentiation from differentiation on collagen IV and from spontaneous differentiation as a comparison.

6.3.3.1. Experimental Outline

RNA samples were harvested from cells differentiating on collagen IV for RT-PCR. RNA was taken immediately before selection was applied (6 days of differentiation), 24 hours after selection was applied (7 days of differentiation), 24 hours after the replating step had been carried out (8 days of differentiation), and two timepoints thereafter (10 and 12 days of differentiation) to see whether markers of multipotentiality, haematopoietic-specific genes and endothelial-specific genes were being transcribed.

RNA was isolated from cells differentiated as a monolayer on gelatin coated plates and from EBs as a comparison for the induction of vascular transcription from directed differentiation on collagen IV.

6.3.3.2. Results

Figure 6.5 shows the relative expression of key genes in vascular development in ES cells differentiated using different differentiation techniques. Samples from cells differentiated in the presence and absence of HAT selection were taken from cells differentiated on collagen IV and used for transcriptional analysis. Robertson *et al.* (2000) observed induction of *brachyury* in 2-day-old EBs, *flk-1* in 2.5-day-old EBs and *scl/tal1* in 3.5-day-old EBs. It is evident from the very low levels of *brachyury* expression that differentiation had passed the early, undefined mesodermal stage when *brachyury* would typically be expected to be expressed. *Brachyury* was not seen in EBs, or in cells differentiated as a monolayer and was only faintly seen in 6-

day-old cultures on collagen IV. Expression of *scl/tal1*, expressed in the earliest stages of haematopoietic commitment was seen. *Pecam-1*, *flk-1* and *flt-1* were all expressed, signifying commitment to the endothelial pathway. *Flk-1* expression was first observed at 2.5 days of differentiation in EBs and 4 days of differentiation for monolayer cultures. By 6 days growth on collagen IV, *flk-1* expression was very strong and in all instances of *flk-1* expression, a similar pattern of *flt-1* induction was also seen. Although expressed more clearly in monolayer cultures, both on gelatin and on collagen IV, *pecam-1* expression was noticeably low in differentiating EBs.

The amplification of transcripts was not successfully standardised using the β-actin housekeeping gene control despite attempts to equalise the cDNA concentration used in each reaction, so direct comparisons of the levels of expression cannot be made. However, there were clear trends for the expression patterns over the course of differentiation. (In order to accurately compare cDNA quantities, real-time RT-PCR could have been employed, had there been more time.) In the collagen IV differentiation, the levels of transcript for all the vascular genes dropped over time. This was not seen in the monolayer and EBs analysed however, these exhibited lower levels of expression for all the genes analysed and only a short period of differentiation was investigated for EB differentiation.

An anomaly was observed with respect to *hprt* expression and its expected correlation with *flk-1* expression (**Figure 6.5**). Although *hprt* expression was seen in all instances where *flk-1* expression was seen for collagen IV differentiation and in EBs, no *hprt* transcript could be amplified from monolayer differentiation, which was peculiar. This result was repeatable and could not be attributed to PCR error or the integrity of a set of cDNA or RNA samples.

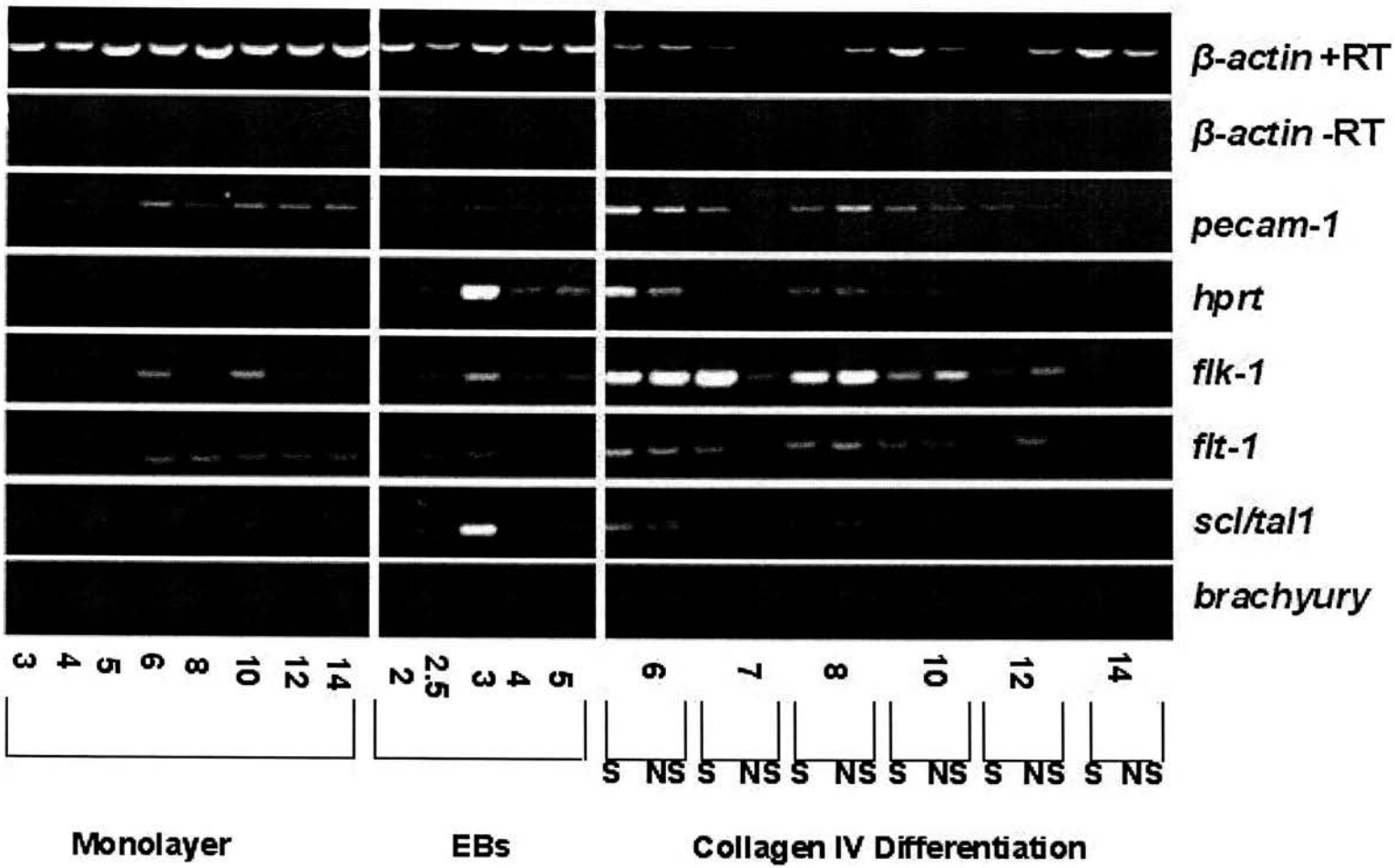

Figure 6.5 *RT-PCR analysis for key genes expressed during mesoderm commitment to the vascular lineages in F8 flk-1/HPRT ES cells being differentiated on a gelatin-coated* ***monolayer****, in suspension culture as* ***EBs*** *and on collagen IV both in selection (****S****) and with no selection (****NS****). Numerical markers indicate the number of days of differentiation. The size of each PCR product for each primer is given in Table 2.1.*

6.3.4. Patterns of Gene Expression during Differentiation on Collagen IV as Detected By Immunohistochemistry

Immunohistochemical staining for FLK-1 (haemangioblastic, endothelial), SCL/TAL1 (haemangioblastic, haematopoietic), VE-Cadherin (endothelial) and αSMA (smooth muscle) were carried out at different stages of differentiation on collagen IV to assess the patterns of differentiation and protein expression over a specific time period. This would add to the RT-PCR results by confirming the expression of the genes of interest at the protein level, and show any patterns of expression that could not be deduced from transcription data.

6.3.4.1. Experimental Outline

A differentiation experiment was set up with collagen IV coated 6-well plates where selection was applied after 6 days of differentiation. 24 hours after the HAT selection was introduced, the cells were trypsinised and replated to eliminate any non-cycling cells that would otherwise be unresponsive to the selective influence of HAT. Cells were fixed and stained 4, 6, 9 and 12 days after differentiation was initiated. The cells taken after day 6 of differentiation were maintained in HAT selection. At each stage when immunohistochemistry was carried out, negative controls i.e. the staining protocol with only the secondary antibodies were included to ensure that any observed staining could not be attributable to non-specific binding of the fluorescently-labelled secondary antibody. See **Appendix E** for examples of negative control samples.

6.3.4.2. Results

At 4 days of differentiation, the cells that had adhered to the collagen IV matrix had just undergone the typical population explosion and spreading that was always seen between 2-4 days of differentiation. The cells that plated down initially proliferated in dense clumps to form three-dimensional small clumps before the typically observed cell growth outwards of the colonies and across the culture surface leaving

more densely packed colony centres. Low level FLK-1 expression was seen across the entire cell population VE-Cadherin was also seen to be expressed. It was expressed in cells that were growing two-dimensionally and had distinctly endothelial morphologies. All VE-Cadherin^{+} cells were also FLK-1^{+}, however, immunostaining confirmed that not all FLK-1^{+} cells were also staining positive for VE-Cadherin (**Figure 6.6**).

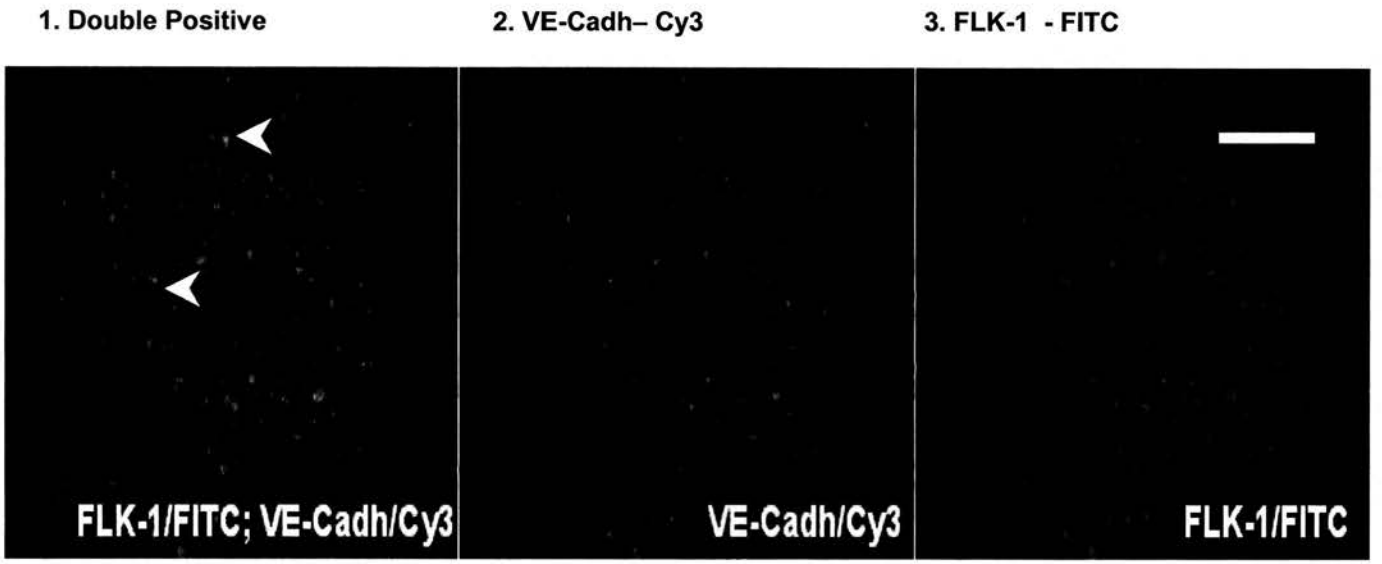

Figure 6.6 *Immunohistological staining for FLK-1 (green/FITC) and VE-Cadherin (red/Cy3) in collagen IV differentiation cultures after 4 days showing co-staining of FLK-1 and VE-Cadherin (**arrowheads**). The FLK-1 staining is shown in the far right column (**3**), the VE-Cadherin staining is shown in the centre column (**2**). The merged images for both epitope stains are shown on the far left (**1**). DAPI nuclear stain is seen in blue. Scale bar represents 100μm.*

By 6 days of differentiation, the cells in 6-well plates were confluent. Colonies differentiating outwards had merged although the foci of each colony remained denser and morphologically uniform compared to the outgrowths. They were generally higher expressers of FLK-1 as well. The FLK-1 staining of the denser foci was not due to an increase in non-specific antibody binding to denser cell masses because there were also dense colonies that did not stain for FLK-1 as well as ones that did (See arrow in **Figure 6.7c**).

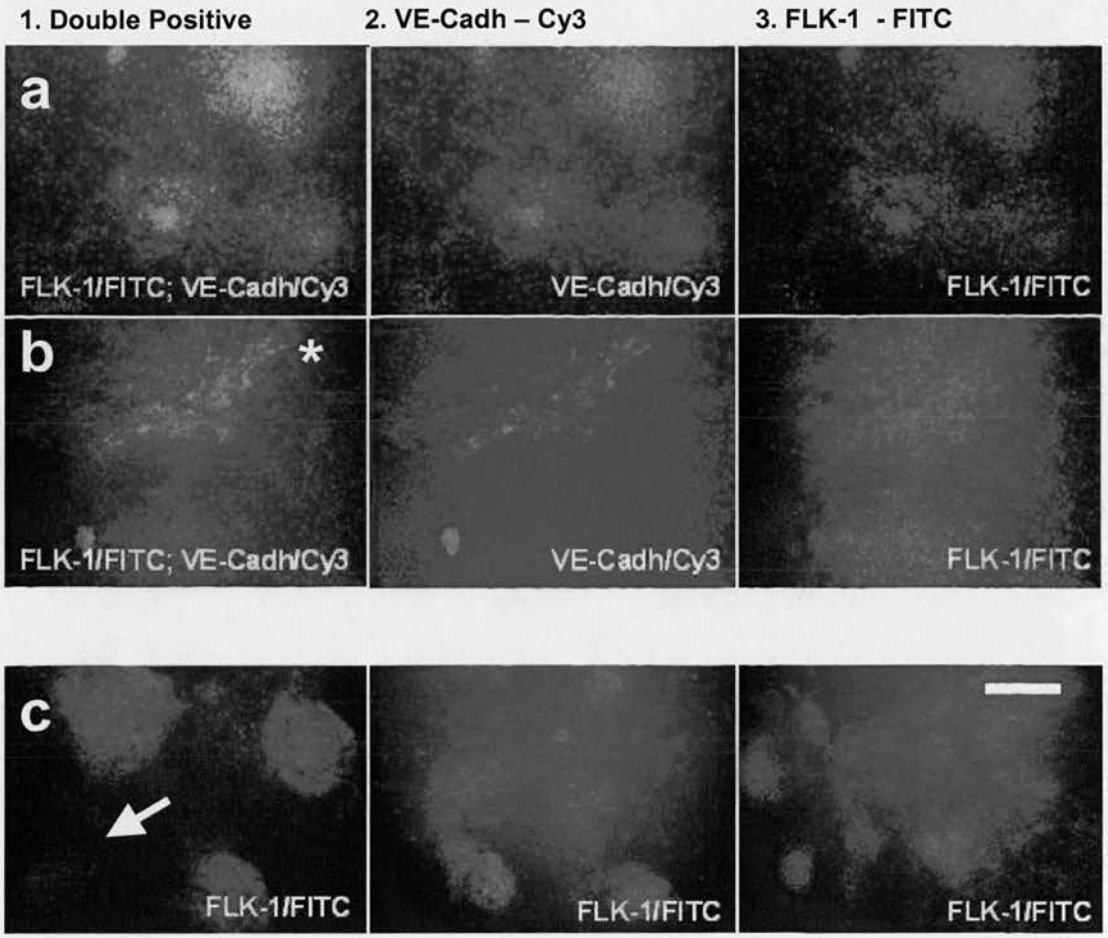

Figure 6.7 *Immunohistological staining for FLK-1 (green/FITC) and VE-Cadherin (red/Cy3) in collagen IV differentiation cultures after 6 days. Rows* ***a*** *and* ***b*** *show co-staining of FLK-1 and VE-Cadherin. The FLK-1 staining is shown in the far right column (**3**), the VE-Cadherin staining is shown in the centre column (**2**). The merged images for both epitope stains are shown on the far left (**1**). The bottom row shows FLK-1 stained cells with varying colony morphologies. The* ***asterisk*** *shows striated coexpression of FLK-1 with VE-Cadherin. The* ***arrow*** *shows a dense colony not staining positive for FLK-1. DAPI nuclear stain is seen in blue. Scale bar represents 100μm.*

Although not expressed across the whole FLK-1 expressing population, levels of VE-Cadherin had also increased by 6 days of differentiation. Some colonies of growing cells exhibited a distinct pattern of VE-Cadherin expression where the central cells of a colony grew very three-dimensionally and stained strongly for FLK-1. On the periphery of these foci, cells grew flatter, expressed FLK-1 and VE-Cadherin and had tight endothelial morphologies. Adjacent to these cells, the cells grew the flattest, more cytoplasmic and more trapezoid in shape. They expressed FLK-1 but not VE-Cadherin. **Figure 6.8** shows examples of the cell morphologies seen in colonies of cells growing on collagen IV.

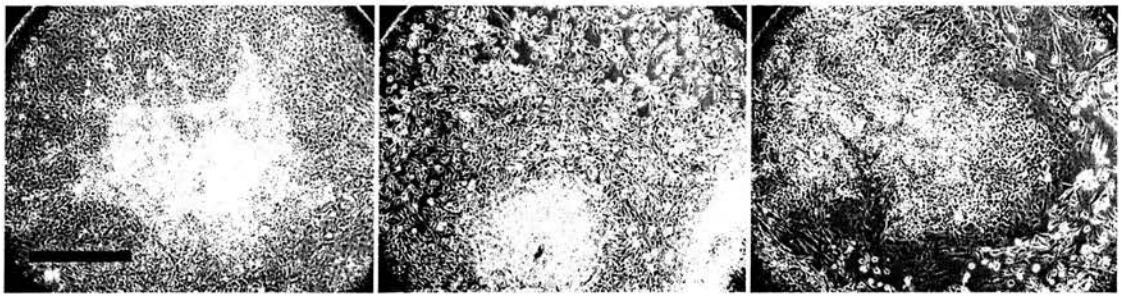

Figure 6.8 *6-day-old colonies differentiating on collagen IV displaying colony structures and the various striations of cell morphology typically seen. Scale bar represents 200μm,*

At day 9 of differentiation, the differentiating cells had been in selection for 72 hours and had been trypsinised and replated 48 hours prior to fixation and staining. Strong FLK-1 expression was seen throughout these plates. The cell surface pattern of staining was consistent with what is typically seen in endothelial cells i.e. discrete but intense staining across the cell surface, giving cells a mottled staining pattern (**Figure 6.9a, b, c**) previously described in FLK-1 staining of endothelial cells (Choi *et al.*, 1998).

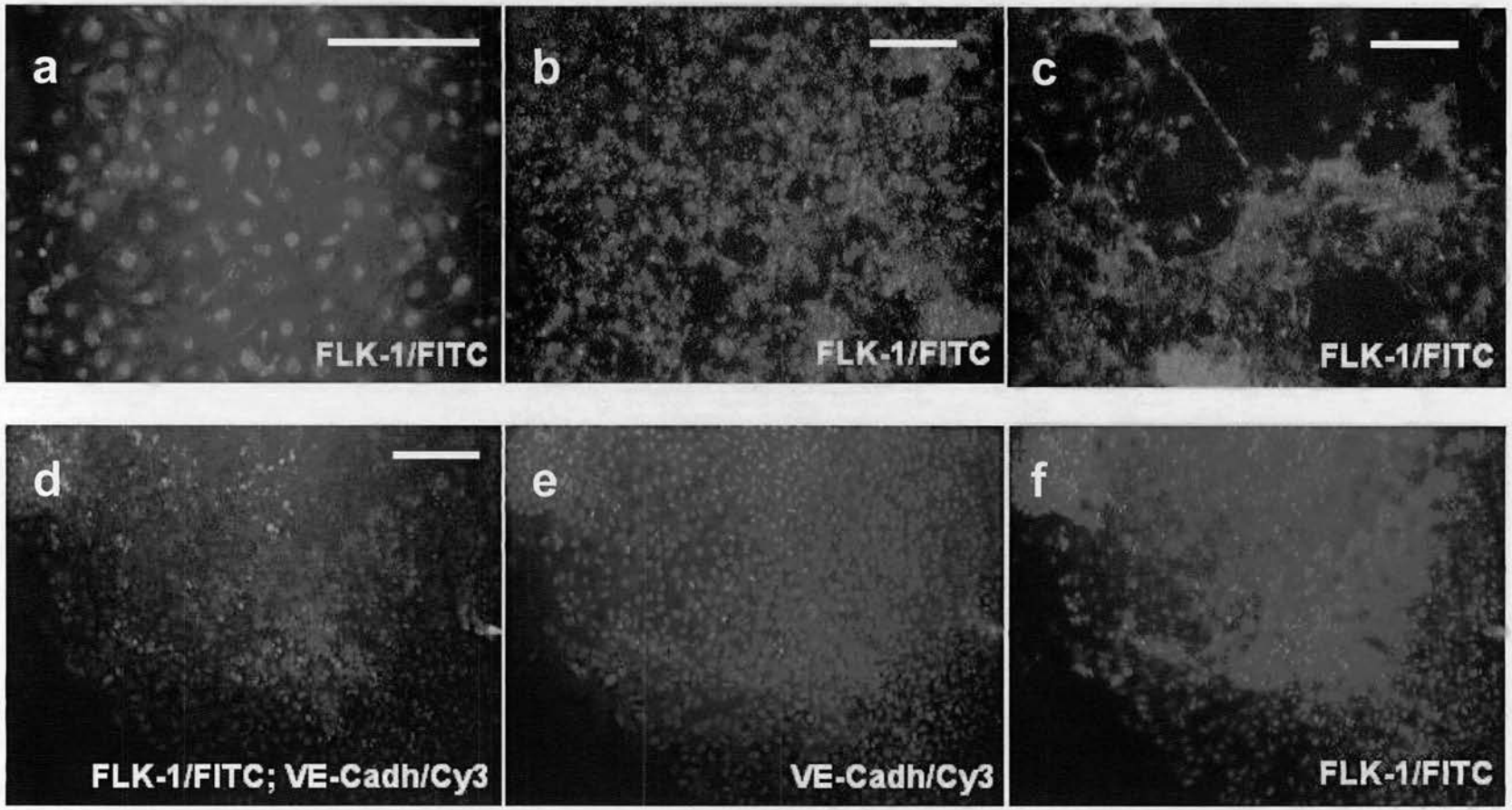

Figure 6.9 *FLK-1 staining (**a, b, c**) and FLK-1 costained with VE-Cadherin (**d**) after 9 days of differentiation. The epitopes stained are shown individually in **e** (VE-Cadherin) and **f** (Flk-1). DAPI nuclear stain is seen in blue. Scale bars represent 100μm.*

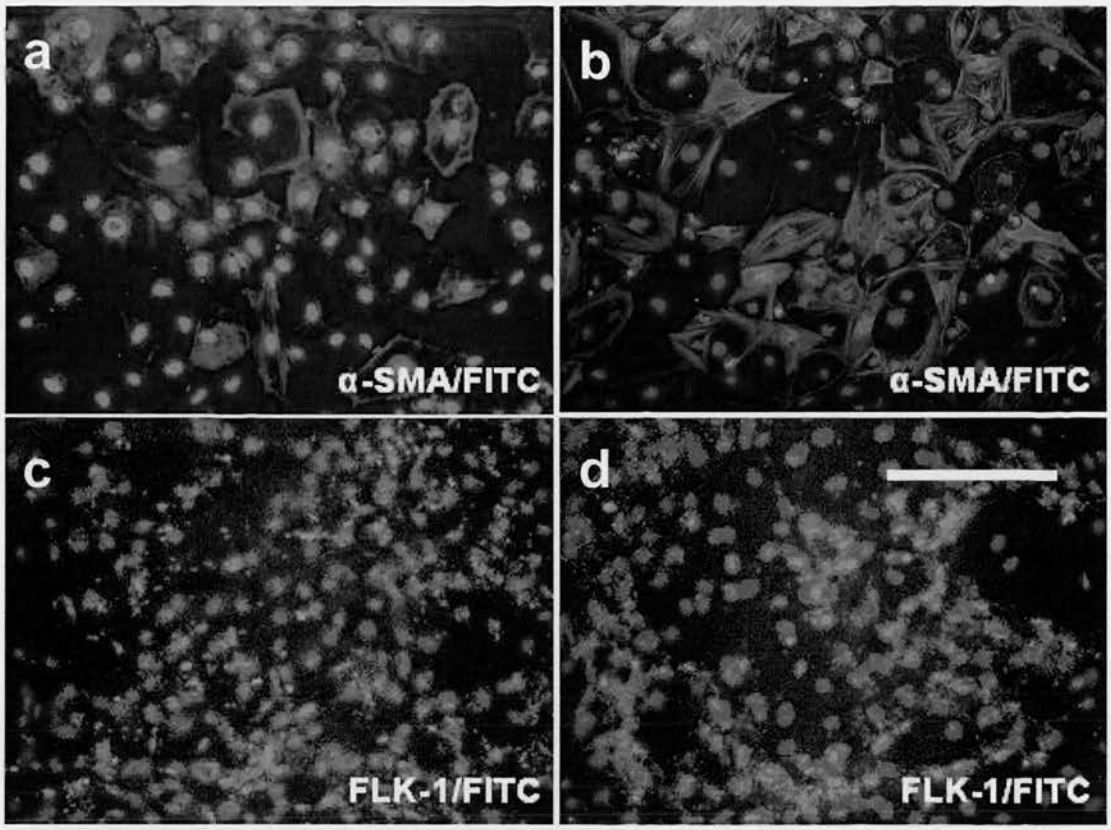

Figure 6.10 *Immunostaining for FLK-1 (**a, b**) and αSMA (**c, d**) after 12 days of differentiation. DAPI nuclear stain is seen in blue. Scale bar represents 100μm.*

By 12 days of differentiation, most of the cells had lost their endothelial morphology. Most were very flat and trapezoid in morphology. They stained strongly for αSMA as well as FLK-1 (**Figure 6.10**). Little cell proliferation was seen. Despite this, upon disaggregation and replating, many cells re-adhered to the collagen IV even though HAT selection was still being maintained. This indicated that FLK-1 was still being expressed to high enough levels to transcribe sufficient HPRT for cells to survive in HAT although cell proliferation was low or undetectable.

6.3.4.3. Summary of Immunohistochemistry Results

From the earliest stages of differentiation, FLK-1 was detectable by immunohistochemistry. At 4 and at 6 days of differentiation, no clear SCL/TAL1 staining could be detected (**Figure s 6.6 and 6.7**) although the antibody against

SCL/TAL-1 was not optimised fully (due to the absence of a positive control SCL/TAL-1 expressing cell line) and was therefore not thought to be reliable. The FLK-1 expression increased by 6 days of differentiation when VE-Cadherin could also be detected. Concentric patterns of staining were seen across the colonies. The central cells were FLK-1^+. These densely packed cells were surrounded by morphologically endothelial cells that stained for both FLK-1 and VE-Cadherin. As the cells lost their endothelial morphology and became more cytoplasmic, they lost VE-Cadherin staining and showed lower FLK-1 staining. This lowered level of staining may have been due to the cells being flatter and more spread out and not related to levels of FLK-1 expressed on the cell surface.

At 9 days of differentiation the cells had been subjected to selection and replating so the colonies had been lost. Most cells stained strongly for FLK-1 and displayed typical mottled endothelial staining patterns (Choi *et al.*, 1998). VE-Cadherin staining was also common and only stained FLK-1^+ cells. Not all FLK-1^+ cells expressed VE-Cadherin however. VE-Cadherin staining was still detectable at 12 days of differentiation, albeit at very low levels. Most cells exhibited muscle cell morphology and stained positive for αSMA while still retaining FLK-1 expression.

6.4. *In Vivo* Transplantation of Purified FLK-1 Expressing Cells

Differentiated and selected FLK-1^+ cells were grafted into a mouse blastocyst to see whether the *in vitro* isolated cell population could contribute to haematopoietic and vascular lineages. It was therefore necessary to track the injected cells. The F8 *flk-1*/HPRT cell line was co-transfected with two plasmids: one contained a blasticidin cassette; the other, pEGFP-C1 (BD Biosciences) contained the EGFP reporter driven by the CMV promoter. The GFP plasmid was transfected at a ten fold higher ratio than the blasticidin cassette to increase the likelihood that any blasticidin resistant cells isolated would have also had the GFP construct transfected and integrated into their genome. Out of the surviving colonies, only very green and thus high GFP expressers were selected.

6.4.1. Experimental Outline

The transfected lines selected were maintained clonally. For *in vivo* grafting assays, they were differentiated for 6 days before selection was applied. At 7 days of differentiation, they were replated and maintained in HT (hypoxanthine and thymine) selection. The aminopterin needed to be eliminated 4 days prior to the injection of these cells back into embryos. The aminopterin would have had its inhibitory effect on the purine biosynthesis pathway for approximately 4 days and would have resulted in cell death *in vivo* in the absence of an immediate source of purines as would be the case if HAT selected cells were grafted directly back into embryos.

The need to wean cells off the aminopterin had the disadvantage of prolonging the culture period required to complete the selection of FLK-1^+ cells and prime the cells for grafting. At least 48 hours of selection followed by replating was required. Adding 4 days of HT maintenance meant that the cells used for injection had undergone at least 10 days of differentiation. It was believed that in order to maximise the enrichment for FLK-1 expressing cells but minimise the terminal differentiation of the cells to endothelial or muscle cells, 9-day-old differentiated cells would give the best and most specific engraftment. Together with the HT weaning period, the minimum time required would be 10 days.

6.4.2. Results

A variety of differentiation and selection regimes were tried during the course of these experiments as summarised in **Table 6.1**.

DIFFERENTIATION (days in differentiation culture)				N° Blastocyst injected and transferred	N° developing at 10 dpc
Selection Applied	Replating	HT Selection	Blastocyst Injection		
6	7	8	12	14	0
6	7	11	15	22	0
6	7	9	13	9	0
6	7	7	9	17	3(1*)

Table 6.1 *Differentiation and selection regimes employed for in vivo transplantation of differentiated FLK-1$^+$ cells. * 1 live birth was harvested 2 days post-birth. 2 neonatal murine pups died.*

In 3 of the 4 grafting experiments carried out, the pregnant mice carrying the blastocyst injected embryos were sacrificed at 10 dpc to harvest the embryos and look at any contribution that the blastocyst-injected cells could have made to the developing foetus. In these experiments, all foster mothers were found to show no signs of pregnancy or were carrying decidua that did not contain embryos, a sign that the pregnancies had been resorbed.

In one experiment, the foster mother was allowed to carry the transferred embryos to birth. Three mice were carried to term. Two mice were dead at birth. The live birth was sacrificed after 2 days and it's internal organs removed and cryopreserved. GFP immunohistochemistry was carried out on the organs.

Figure 6.11 shows the results of the antibody staining of the organs that were successfully removed and stained for the GFP protein with a FITC-conjugated GFP antibody. Some organs infiltrated by a complex and broad vascular network were found to show a high level of staining, however others, such as the brain which is

also highly vascularised were found to only have peripheral staining. Wild-type tissues showed no staining with the GFP antibody (data not shown). The staining pattern did not show localisation of the injected cells to the vascular lineages indicating that the cells were either not restricted to the vascular lineages or that they had been reprogrammed to take on other cell fates. The latter may be the less likely possibility of these two, however, given that the cells injected would be likely to be equivalent to cells from a later developmental timepoint than those in the blastocyst in which they were injected. A lack of time prevented further differentiation and grafting experiments being carried out or double staining for vascular markers to be carried out to distinguish the location of the vasculature in the tissue sections and the relative locations of the GFP-marked cells.

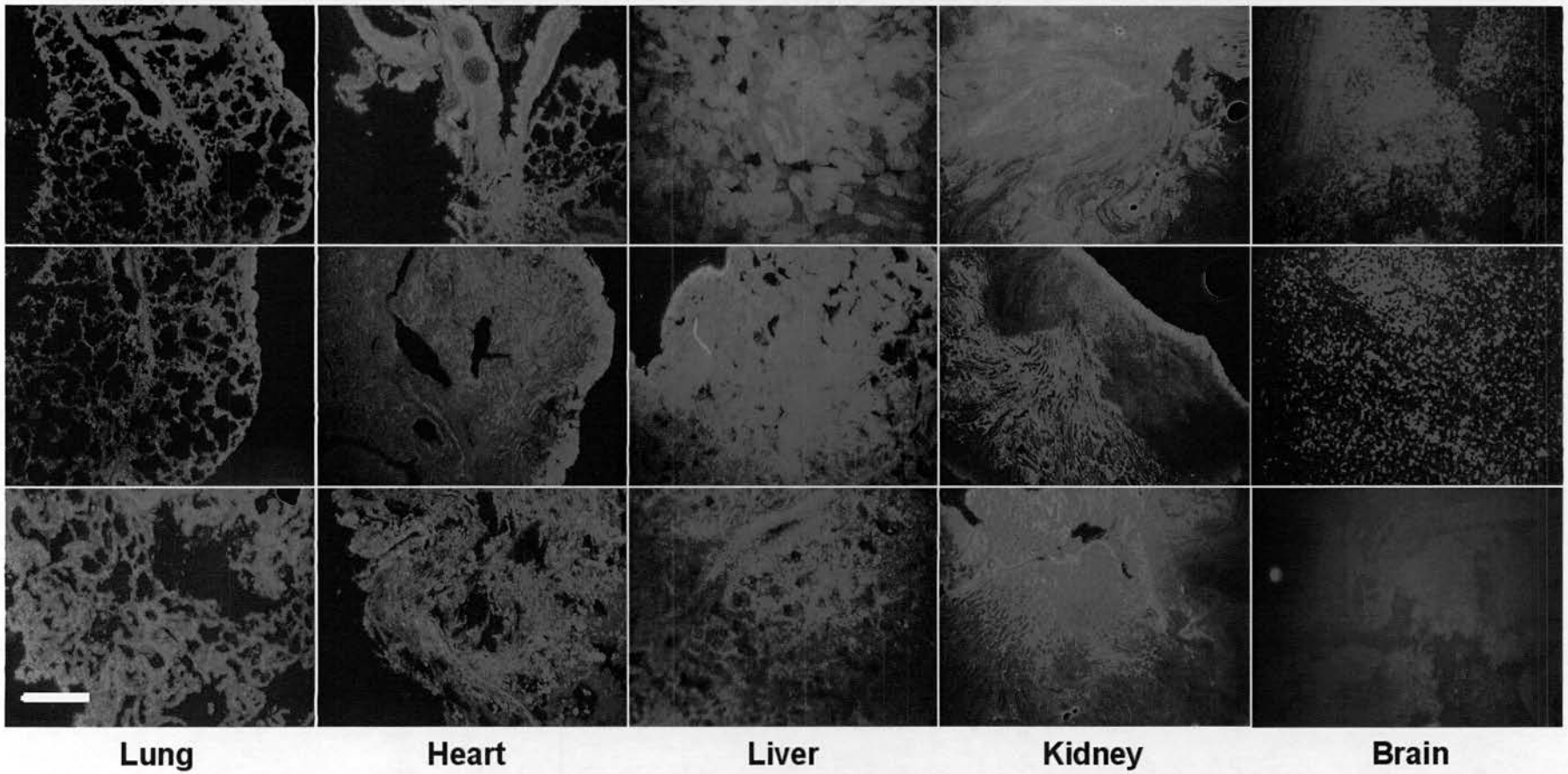

Figure 6.11 *GFP immunohistochemistry of cryosectioned organs from a 2-day-old neonatal mouse following blastocyst injection with purified FLK-1 expressing differentiated ES cells. DAPI nuclear stain is seen in blue. Organs were sectioned longitudinally. Wild-type tissue showed no green fluorescence. Scale bar represents 100μm.*

6.5. Discussion

6.5.1. Optimisation of Collagen IV Differentiation and Selection Regime

The Nishikawa protocol for the differentiation of FLK-1$^+$ cells (Nishikawa *et al.*, 1998a) was modified to optimise the time at which selection was applied. It was found that unlike the observation of Nishikawa and colleagues, where FLK-1 is induced almost immediately after plating on collagen IV, HM1-derived ES cells plated at the recommended low density, took several days before growth and differentiation were observable. The selection regime needed to be introduced as soon as possible if the FLK-1$^+$ cells isolated were to be progenitor cells and not terminally differentiated endothelial cells. It was found that the earliest time that selection could be applied without the death of all cells was 6 days after plating on collagen IV.

The observation was made in the HM1 parental ES cell line, that the selection procedure, though highly effective, did not kill all of the differentiating HM1 cells. HAT selection only has an effect on cycling cells. As endothelial cells are able to enter quiescence (Witmer *et al.*, 2002), and senescence is also a common problem associated with differentiated cells *in vitro*, in order to obtain as pure a FLK-1$^+$ cell population as possible, a replating step was introduced into the differentiation protocol to eliminate non-cycling cells. The replating step was found to be effective after being introduced as little as 24 hours after selection was applied.

After replating, the cells grew with uniform endothelial-like morphologies. Spherical refractile cells were seen to bud off them and a steady although slow rate of proliferation was observed for up to a week after the replating step was performed. These observations hinted that the cells being purified were haemogenic endothelial cells.

Transcriptional analysis using RT-PCR over the course of collagen IV differentiation (**Figure 6.5**), showed that by 6 days of differentiation, key vascular genes were being

transcribed in the adherent cells including *scl/tal-1* which, being an early haematopoietic and haemangioblastic marker, would not necessarily be associated with the adherent cells, were they already terminally differentiated to endothelium. This indicated that at these earlier stages of differentiation, either haematopoietic precursors were adhering to the adherent cells (possibly supporting the haemogenic endothelium theory) or that the adherent cells were haemangioblastic themselves, co-expressing *flk-1* and *scl/tal-1*. As differentiation progressed, expression levels dropped for all of the vascular markers until at 14 days of differentiation, none could be detected. In the absence of exogenous growth factors to stimulate growth, progenitor maintenance or further differentiation to vascular lineages, it is not surprising that expression was lost. *Pecam-1* expression showed the same downregulation over time that was seen for the other genes.

Monolayer differentiation showed the lowest levels of vascular gene induction out of the three modes of differentiation that were analysed except for pe*cam-1* induction which was detected at very low levels in EBs. This hints that there may be cellular interactions on cells growing as a monolayer that are not present or which are inhibited in three-dimensional EBs or vice versa. Another notable outcome was the inability to detect *hprt* transcription in monolayer differentiation on gelatin. As the *flk-1* transcripts were found to be less highly expressed in the monolayer differentiation in comparison with the in EBs and collagen IV differentiation, it was possible that the absence of *hprt* transcript was a sensitivity issue with the PCR. There was a clear peak in vascular transcripts at 3 days of EB differentiation, which corresponds with the Keller observations of the haemangioblastic window in differentiating EBs (Kennedy *et al.*, 1997; Choi *et al.*, 1998).

6.5.1.1. Immunohistochemical Patterns of Expression During Differentiation

The data obtained from immunohistochemical staining of differentiating ES cells helped to visualise the patterns of differentiation. Early on in the differentiation procedure, SCL/TAL1 and FLK-1 were seen to be expressed by clustered cells in the middle of outwardly differentiating colonies of cells. By 6 days, a clear colony

structure had developed with central, densely packed and refractile FLK-1$^+$ cells being surrounded by endothelial FLK-1$^+$ VE-Cadherin$^+$ cells followed by a muscle cell-lie peripheral ring of FLK-1$^+$ cells (**Figures 6.7 and 6.8**). There were clear similarities to blood vessel morphology and structural order and may hint that a similar order and pattern of differentiation is maintained during vasculogenesis or angiogenesis whereby there exist cells displaying haemangioblastic characteristics including expression of FLK-1 and SCL/TAL1. Haematopoietic cells may bud off these cells and FLK-1$^+$ VE-Cadherin$^+$ cells grow out from them. Peripheral to these lay flatter cells similar to muscle cells (**Figure 6.12**).

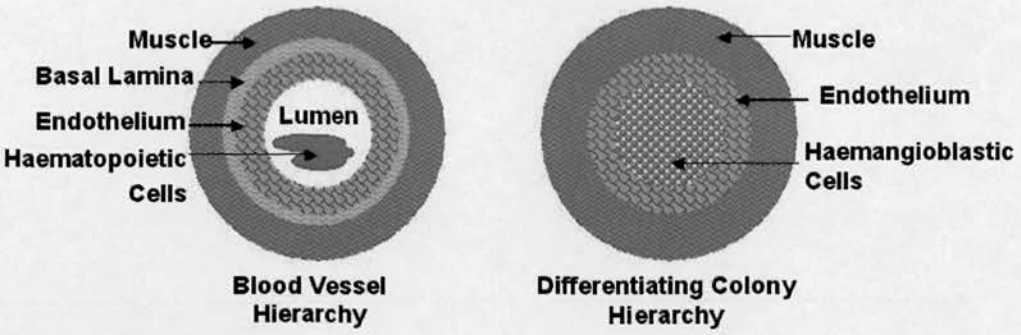

Figure 6.12 *Schematic diagram showing the similar hierarchy of cell layers in blood vessels and cells differentiated on collagen IV in vitro.*

At 9 days, after the replating step to eliminate noncycling cells, the colony hierarchy was lost and flat sheets of FLK-1$^+$ cell remained. Most of these stained for VE-Cadherin and were therefore confirmed to be endothelial. It was clear that by 9 days of differentiation, most cells were no longer haemangioblastic in appearance and displayed signs of lineage restriction to the endothelial lineage, both morphologically and according to their expression profiles. By day 12, two distinct cell morphologies were seen: the FLK-1$^+$ endothelial-like cells were still present, although VE-Cadherin was not detectable at very high levels, and αSMA$^+$ muscle-like cells. This may be attributable to the lack of direction given to the cells beyond the enrichment for FLK-1. Perhaps in the absence of a follow on signal, these differentiating cells may be unable to proliferate for an extended length of time in culture.

A concern regarding the selection regime being applied to the cells was the removal of a cellular hierarchy that could have been acting as an *in vitro* niche with certain FLK-1$^-$ cells supporting FLK-1$^+$ cells, possibly even promoting their proliferation and maintenance in an undifferentiated state. The influence of growth factors may have powerful stimulatory influences on the cell signalling mechanisms that drive differentiation (reviewed in Czyz and Wobus, 2001; Burdon *et al.*, 2002). However, alternative forms of activation via mechanoreceptors and adherence to extracellular matrices and adhesion molecules also exist (Chen *et al.*, 1999; Jalali *et al.*, 2001; Wang *et al.*, 2002). Although there is redundancy in the modes of activation of various signalling cascades, it is likely that transcriptional activation via integrins and other adherence molecules could significantly affect the fate and activity of a pluripotent or differentiating cell. Of course, the reverse scenario was also possible with non FLK-1 expressing cells promoting terminal differentiation of FLK-1$^+$ cells or redirection away from vascular cell fates, meaning that the removal of such cells would be an advantage.

6.5.1.2. *In Vivo* Potentiality *of In Vitro*-Derived Differentiated Progenitors

To address the concerns regarding the removal of a niche-like hierarchy with the HAT selection and the effect this may have had on the *in vitro* derivation of a haemangioblastic cell population, these differentiated cells were transplanted back to the blastocyst stage embryo. Even if haemangioblastic cells had successfully been isolated from ES cells, these cells being injected into the blastocyst were at a developmentally more advanced stage than the embryo in which they were intended to integrate. Several different experiments were carried out, however, most of the blastocyst transfers did not survive to 10 dpc when they were to be harvested. In one experiment, the pregnancies were allowed to go to term. The only live birth was sacrificed after 2 days and its organs analysed for contribution from the GFP labelled and differentiated cells. All highly vascularised organs that were looked at showed some staining for GFP and had therefore been infiltrated by the differentiated ES cell-derived cells. The brain only showed a small level of expression peripherally. It was difficult to determine the lineage specificity of the cells from the staining

patterns. The GFP^+ cells did appear to be highly localised to the endothelial or haematopoietic lineages, however all the organs examined were highly penetrated by the vasculature and due to fenestration (permeability of the endothelial layer) of the endothelial cells in these organs, blood lineages could freely enter the tissues. It was unfortunate that there was no time to successfully repeat the grafting experiments and stain for markers from the vascular lineages as well as for GFP to determine whether the *in vitro*-derived cells were maintaining a vascular identity or if they were contributing to other lineages.

Even if they were not vascular-specific in their lineage specifications *in vivo*, the transplanted cells were differentiated beyond the time of expression of markers of more plastic cell status e.g. *brachyury* and had been differentiated in the absence of LIF for 9 days. They had been maintained in the blastocyst despite their more advanced stage of differentiation and were able to contribute to many lineages. If studies could have been extended, it would have been interesting to see whether these cells were vascular and if they were not, whether they were restricted to mesodermal tissues and organs. Many more questions regarding reprogramming, the possibility of cell fusion, dedifferentiation and transdifferentiation arise from these *in vivo* results and would have benefited from the execution of further *in vivo* studies.

6.6. Summary

- The haemangioblast differentiation protocol on collagen IV was optimised for the highest and quickest yield of HAT resistant cells which were assumed to be $FLK\text{-}1^+$.

- These cells were seen to colonise highly vascularised organs although it was not determined whether they were specific to cells of the vasculature or had differentiated to other lineages.

CHAPTER 7

Concluding Remarks and Future Directions

Concluding Remarks and Future Directions

7.1. Introduction

There is a broad range of potential applications for stem cells especially the more versatile ES cell. ES cells enable the study of development and differentiation at the molecular level *in vitro*, they enable the generation of precisely designed transgenic mice to model disease and could be used to determine the effect and efficacy of chemical components or drugs before the requirement for *in vivo* studies in animal models and clinical trials. The most desirable and powerful application of stem cells focuses on their pluripotency and their possible use to facilitate the establishment of cell-based therapies for disorders resulting in the degeneration of tissue or the loss of cell function. In theory, any disorder could be treated, the limiting factor being the ability to differentiate the appropriate cell type.

This project was designed to investigate the possibility of deriving a progenitor population of cells from ES cells *in vitro* and seeing whether this *in vitro* derived population could be grafted *in vivo* and recapitulate the function for which it was intended.

Mimicking *in vivo* events through the differentiation of ES cells has been possible (Doetschman *et al.*, 1985) and has been a useful tool for investigating the signalling events that govern differentiation in the developing embryo (reviewed in Czyz and Wobus, 2001; Burdon *et al.*, 2002). It is unlikely that the true complexity of cellular interactions and signalling will be deciphered or applied to *in vitro* differentiation methods; the aim of *in vitro* differentiation is therefore to generate a cell population that is similar enough to its *in vivo* counterpart to be recognised and captured by the appropriate machinery once grafted *in vivo*, and directed down the appropriate lineage for further differentiation and specification.

The haemangioblast seemed like the ideal candidate as the chosen progenitor. It has been thought for many years to exist but has never been isolated *in vivo*. Promising investigations in chick development hint at its existence (Martin *et al.*, 1980; Pardanaud *et al.*, 1996; Tavian *et al.*, 1996; Jaffredo *et al.*, 1998; North *et al.*, 1999; Ogawa *et al.*, 2001), however, difficulty in accessing the murine embryo at 6-7 dpc, the time of the supposed emergence of the haemangioblast, means that it has never been located in the mammalian model. A cell that is reminiscent of the haemangioblast in its function has been isolated through the *in vitro* differentiation of ES cells, namely the BL-CFC (Kennedy *et al.*, 1997; Choi *et al.*, 1998). Other differentiation studies lend their support to the hypothesis that the haemangioblast hypothesis should at least incorporate the possibility that the endothelium of the forming vasculature has haemogenic capabilities (Nishikawa *et al.*, 1998a; Nishikawa *et al.*, 1998b; Yamashita *et al.*, 2000; Hirai *et al.*, 2003).

By marking ES cells with a reporter gene allowing for the identification of the haemangioblast in culture, isolation of a suitable population of haemangioblastic cells and injection into a blastocyst were to be carried out to test the *in vitro*-derived cells' abilities to reconstitute the haemangioblastic lineages. (When this project was initiated, the plan had been to generate embryonic lethal *flk-1*$^{-/-}$ blastocysts for progenitor injection to see whether the lethality could be overcome by the transplanted cells) The success of such an experiment would verify the possibility of deriving a progenitor population *in vitro* that was functionally active *in vivo* and would also show that cells at a more advanced stage of development could be supported in the blastocyst-stage embryo and be maintained until the appropriate stage of development for their direction to an appropriate function as has previously been shown with HSCs (Muller *et al.*, 1994).

7.2. Gene Targeting at the Flk-1 Locus

The *flk-1* gene is known to mark the earliest mesodermal cells that are specified to the haemangioblastic cell fates. Though not the sole marker of the haemangioblast, it was the best candidate as the marker to isolate a cell population for analysis and transplantation. Two targeting strategies utilised, placed an EGFP reporter or an

hprt minigene under the transcriptional control of the *flk-1* promoter. The GFP reporter would allow for the optimisation of *in vitro* differentiation protocols for maximal *flk-1* expression as well as FACS sorting of a FLK-1$^+$ cell population whilst the *hprt*-targeted cells could be selected for in culture as the ES cell line targeted was otherwise HPRT deficient (Selfridge *et al.*, 1992).

After a series of problematic targeting experiments, the two targeting strategies were successfully carried out. Further investigations aimed at trying to deduce the reasons for the very low targeting frequencies observed were ultimately inconclusive although the possibility that isogenicity of DNA in the arms of homology incorporated in the targeting vectors remained a possible explanation.

7.3. Silencing of the GFP Transgene

Unfortunately, after a few preliminary differentiation experiments, it became apparent that in the *flk-1*/GFP targeted cell lines, the EGFP transgene was being silenced. RT-PCR and Western blotting confirmed that the silencing was occurring at the post-transcriptional level. The cause of loss of transgene expression was never resolved. However, it highlighted the necessity to carefully consider vector design and the genetic locus being targeted. As both of the isolated *flk-1*/GFP targeted lines were silenced, the problem may have been due to the design of the targeting vector and possibly a response to there being two polyadenylation signals in the targeted *flk-1* allele. This may have caused the mRNA sequence to be subject to nonsense-mediated mRNA decay (Maquat and Carmichael, 2001). In hindsight, measures should have also been taken to excise the PGKneo to protect against any silencing induced by its presence (Ramirez-Solis *et al.*, 1993; Rijli *et al.*, 1994; Fiering *et al.*, 1995; Olson *et al.*, 1996; Weissmann & Aguzzi, 1999).

Others have also reported a noticeable improvement in transgene expression when introns were incorporated into the transgene (Fehling *et al.*, 2003). This may explain why the HPRT transgene was expressed in cell lines but the GFP transgene was not. The former had incorporated introns whereas the GFP transgene was a cDNA.

7.4. Differentiation of ES Cells for FLK-1 Expression

The *flk-1*/GFP cell lines were abandoned in the differentiation experiments although several chimaeric mice and heterozygous mutants (which also failed to show GFP expression) were generated from the cells. Differentiation experiments were continued using the *flk-1*/HPRT targeted cell lines. Two very different differentiation protocols were tried out. The Keller protocol generated the suggested *in vitro* equivalent of the haemangioblast: the BL-CFC (Kennedy *et al.*, 1997; Choi *et al.*, 1998) and the Nishikawa protocol that derived a monolayer culture of endothelial-like cells which also had haematopoietic and mural cell potential, supporting the haemogenic endothelium hypothesis.

Optimising the Keller regime was found to be highly problematic. Varying cell density, timing of the BL-CFC culture assay and the method of production of the conditioned medium, which was incorporated into the assay, did not affect the efficacy of the protocol. However, the method of disaggregation of EBs before the setting up of the BL-CFC culture regime was found to be important, with dispase disaggregation resulting in a better yield of BL-CFCs than trypsin, probably due to dispase's more specific protein targets for lysis. Blast colonies derived from BL-CFCs were successfully differentiated to endothelium, haematopoietic precursors and muscle cells as seen after further differentiation on Matrigel and immunohistochemistry and in haematopoietic assays in methylcellulose. Unfortunately, due to the requirement for several lengthy optimisation steps to successfully carry out this culture technique, there was not sufficient time to successfully optimise a selection regime to isolate $HPRT^+$ cells via HAT selection. Considering the variability and the difficulties encountered with this differentiation technique, the possibility to successfully select a pure FLK-1^+/$HPRT^+$ cell population seems unlikely.

Although it was shown that the blast colonies arising from BL-CFCs had the potentiality expected of the haemangioblast, it cannot be concluded that the BL-CFC is the *in vitro*-derived haemangioblast. It would be interesting to see whether BL-CFCs can be directed to other cell fates if different cytokines are applied to the blast colonies derived from BL-CFCs. This line of investigation relies on the ability to isolate BL-CFCs from differentiating EBs, which is, at present, limited by the inability to fully characterise the putative haemangioblast. Initial trials with cells sorted for FLK-1 and SCL/TAL-1 could act as a starting point for these experiments as well as further characterisation of the haemangioblast.

7.5. Isolation of FLK-1 Expressing Cells

The Nishikawa differentiation protocol was far more successful from the outset. Cells were grown as a monolayer on collagen IV coated plates; there was no need for the formation of three-dimensional EBs to stimulate differentiation suggesting that the collagen IV matrix was a sufficient stimulus for differentiation. Also, there was no need for added cytokines unless the yield of FLK-1^+ cells from differentiation on collagen IV was to be specifically directed for differentiation to a particular lineage e.g. endothelium (Yamashita *et al.*, 2000). As the earliest committed progenitors were desirable in this project, such stimulation was omitted. The HAT selection for FLK-1^+ cells was successfully incorporated into this differentiation protocol. A passaging step was also incorporated to eliminate the presence of non-cycling cells that were rendered non-responsive to the effects of HAT. After selection of FLK-1^+ cells was deemed effective, the cells were weaned off aminopterin in preparation for blastocyst injection. Several attempts were made to inject selected FLK-1^+ cells into blastocysts; only one case resulted in a live birth. The neonatal mouse showed colonisation of the HAT-selected and differentiated (and subsequently GFP-tagged) FLK-1^+ cells in some vascularised organs e.g. kidneys, liver and heart, but not in others e.g. the brain – a highly vascularised organ. Unfortunately, there was not enough time available to continue with the *in vivo* colonisation studies. Also, the specificity of the colonised cells could not be determined as there was not enough tissue material available to costain the GFP^+ cells found in the mouse organs for vascular markers to determine the fate of the transplanted cells.

Migrating endothelial cells are usually accompanied by non-endothelial cells e.g. pericytes, smooth muscle or fibroblasts (Nicosia and Ottinetti, 1990; Nehls *et al.*, 1992). Isolation of a pure population for *in vivo* transplantation may not matter if selection is immediately followed by grafting e.g. after FACS for cells expressing a specific set of desirable markers; however, removing such companion cells could have detrimental effects in the middle to long term i.e. whilst cells are being cultured under selection as these cells could have signalling or conditioning properties essential to the survivals of progenitor, early vascular or non-quiescent cells. The removal of FLK-1$^-$ cells via HAT selection, could therefore equate to the elimination of a niche or a cellular hierarchy during differentiation that could impact on the viability of the cell population that is being isolated. It strengthens the argument for the use of flow cytometry for the isolation of cells.

7.6. Possibilities For Further Investigation

As well as continuing the transplantation experiments with the HAT-isolated cell population, several avenues of investigation would be beneficial to pursue to develop this body of work. The HAT-selected cells following differentiation did express FLK-1 and did contribute to embryonic organs following injection into the blastocyst. However, by the time they were harvested for injection, their proliferative capacity had dropped and the majority of cells appeared endothelial in morphology. Though still expressing FLK-1, it is possible that these cells were not haemangioblastic, but endothelial. With the progress made in FACS, specific cell types can be purified from heterogeneous populations with ease, as long as antibodies can be used to bind to the cells. FLK-1 is a cell surface marker that can bind fluorochrome labelled antibodies for FACS for a pure FLK-1$^+$ cell population without the need to adopt complicated transgenic methods. However, incorporation of transgenic strategies with sorting for desirable cell surface markers could be utilised to speedily isolate progenitor populations for therapeutic transplantation. This has already been achieved by Chung *et al.* (2002). The haemangioblastic SCL/TAL-1 transcription factor cannot be sorted for. By targeting the SCL/TAL-1 genetic locus with a non-functional CD4 cell surface marker, they enabled

SCL/TAL-1 expressing cells to be isolated via the expression of this epitope. In combination with sorting for FLK-1 directly, a clever strategy to further study and characterise a putative haemangioblast population was developed. This needs to be further built upon to gain a better understanding of the haemangioblast and its role in development. The adoption of a similar strategy to further characterise the putative haemangioblast would be interesting, although dependent on the selection of a reliable candidate gene for targeting that is expressed early in vascular specification but not preferentially expressed in any one of the different vascular cell fates.

It has yet to be proved definitively that the haemangioblast exists *in vivo*, though there is much *in vitro* and *in vivo* data to suggest that it is likely to exist and the criteria for identification of progenitors and their subsequent descendants are always contentious issues. An array of different cell surface markers are used to identify pre-haematopoietic mesoderm and haematopoietic or endothelial precursors. It is common to find conflicting data suggesting that the expression of a marker one group has used to purify a supposedly pure haematopoietic progenitor population is actually heterogeneously expressed in a pure haematopoietic progenitor population isolated by another group based on expression of a different cell surface marker e.g. the discovery that $CD34^-$ LTR-HSC exist (Osawa *et al.*, 1996; Ando, 2001). It is therefore likely that several candidate markers that are expressed around the time that the vascular system is established will need to be evaluated with care to identify a likely marker of a haemangioblastic cell.

7.6.1. Extrinsic Influences on Vascular Differentiation

As mentioned, the process of selection was flawed due to the lag time taken for selection to take effect and a pure population to be isolated. It may be possible to speed up commitment to the haemangioblastic lineages, without terminal differentiation to a specific cell fate by considering the application of extrinsic influences on ES cells *in vitro*. Many *in vitro* differentiation experiments carried out to try to understand the molecular mechanisms that underlie the cell specification in ontogeny do not address the role of the specific microenvironment on the potentiality

and maintenance of a progenitor. The *in vitro* culture and manipulation of cells, particularly ES cells used to recapitulate the actual mechanisms of lineage determination, are limited by the crudeness of the experimental systems that are used to mimic *in vivo* signalling and cell fate determination. However, extrinsic influences could be a crucial element in the mimicry of *in vivo* events and potentially, a niche hierarchy. Such stimulation is also integral to the development of cell-based therapies if they are to be robust and accepted at the graft site after transplantation.

The extracellular matrix and shear stress stimulate the differentiation and maintenance of progenitors. *In vitro*, endothelial cells have been shown to respond to shear stress with increased cell division and motility at the sites of flow separation (Tardy *et al.*, 1997). Incorporation of shear stressors, the use of scaffolds, the creation of a hypoxic environment along with the application of appropriate cytokines with the HAT selection regime would all have been interesting to investigate concurrent with the differentiation and selection of the FLK-1 selectable cells to see if they enhanced the yield and maintenance of an early progenitor population.

Levenberg *et al.* (2002) isolated endothelial cells from spontaneously differentiated hES cell-derived EBs using FACS isolation with the PECAM1 antibody. These cells formed vessel-like structure *in vitro* and proliferated to form vessels when transplanted into SCID mice supported by either a poly-L-lactic acid or a polylactic-glycolic acid scaffold. These *in vivo* generated vessels were found to harbour murine haematopoietic cells in their lumen. Not only is this a promising example of the potential use of hES cells in therapy but also supports the theory that endothelial cells have haemogenic capabilities.

Niklason *et al.* (1999) used biodegradable polymer scaffolds and bioreactors simulating *in vivo* conditions to successfully grow blood vessels from cells taken from bovine aorta. The use of the bioreactor induced inward migration of SMCs, typical of native blood vessels. This was not observed in cultures maintained outside

a bioreactor but with otherwise identical growth measures. This highlights the importance of extrinsic influences in the development of therapeutically viable and structurally correct tissues for transplantation.

7.7. *In Vitro*-Derived Cells as Therapies

BM-derived endothelial progenitors have been previously isolated (Takahashi *et al.*, 1999) and can be recruited for repair and generation of vessels in ischemic or damaged tissues, tumours and even non-damaged tissues (Asahara *et al.*, 1999).

Asahara *et al.* (1997) isolated $CD34^+$, $FLK\text{-}1^+$ cells from human peripheral blood. They injected this population of cells into the tail vein of athymic nude mice that had had their femoral artery removed and hence had ischaemia in the hindlinb. The peripheral blood cells successfully differentiated to endothelial cells that revascularised the ischaemic hindlimb showing that haemangioblastic or endothelial progenitors existed in adult peripheral blood. More recently vascular structures have been differentiated from hES cells with a protocol based on differentiation on collagen IV (Gerecht-Nir *et al.*, 2003). Levenberg *et al.* (2002) successfully injected their isolated endothelial cells into mice where they formed seemingly functional microvessels.

These are encouraging achievements in the development of vascular cells with potential therapeutic applications. As the molecular mechanisms underlying ES cell self-renewal and differentiation are disseminated, a greater understanding of the signalling mechanisms required for specific lineage differentiation will hopefully allow more specific cell types and progenitors to be isolated *in vitro* for therapeutic applications.

CHAPTER 8

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ZHAO L.R., DUAN W.M., REYES M., KEENE C.D., VERFAILLIE C.M. and LOW W.C. (2002) Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. *Experimental Neurology* **174**, 11-20.

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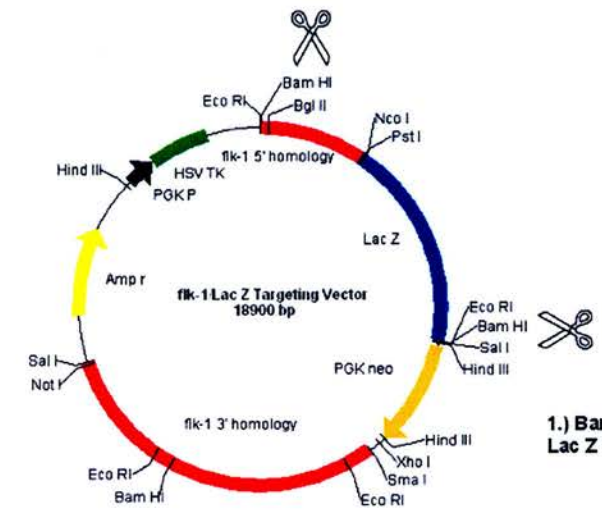
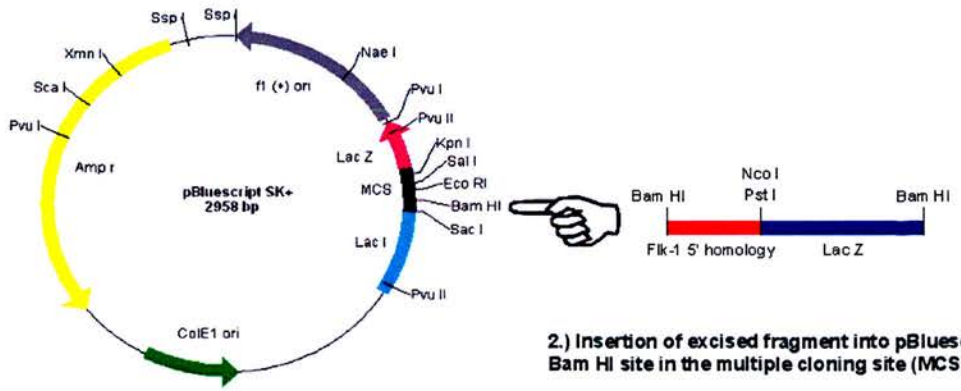
CHAPTER 9

Appendices

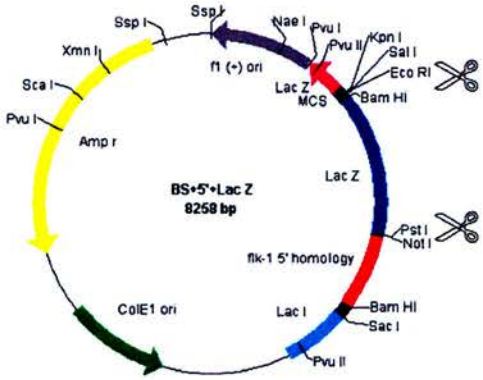
Appendix Ai:
Cloning Strategy: flk-1/HPRT Vector Construction



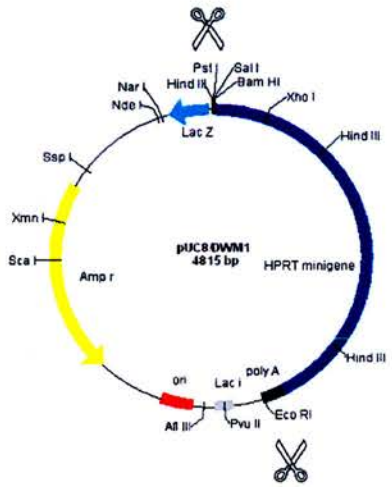
1.) Bam HI digest to excise 5' homology and Lac Z reporter



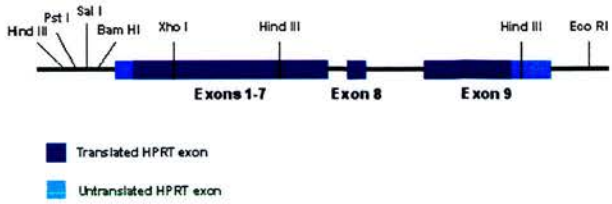
2.) Insertion of excised fragment into pBluescript SK+ at Bam HI site in the multiple cloning site (MCS).



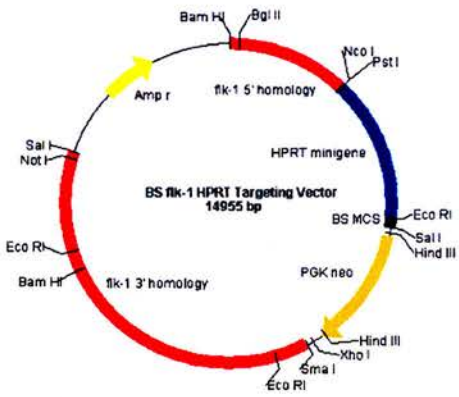
3.) Excision of Lac Z reporter using Eco RI and Pst I enzymes.



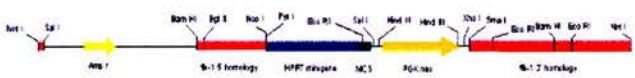
Structure of the HPRT Minigene



4.) Concurrently, the HPRT minigene was excised from the DWM-1 plasmid using Pst I and Eco RI

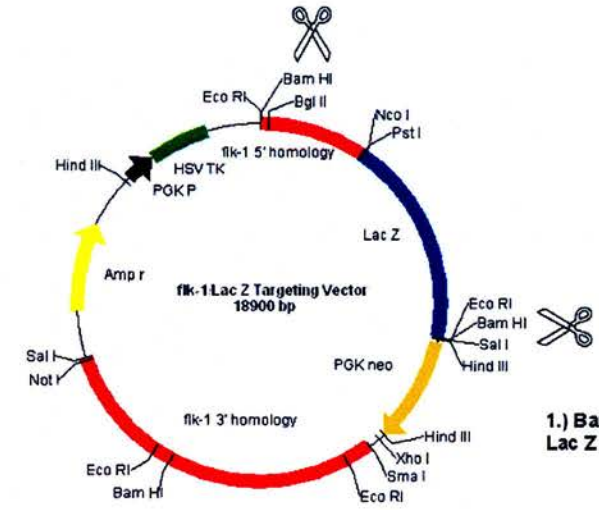


5.) The isolated HPRT fragment was used to replace the excised Lac Z reporter to complete the *flk-1*/GFP plasmid.

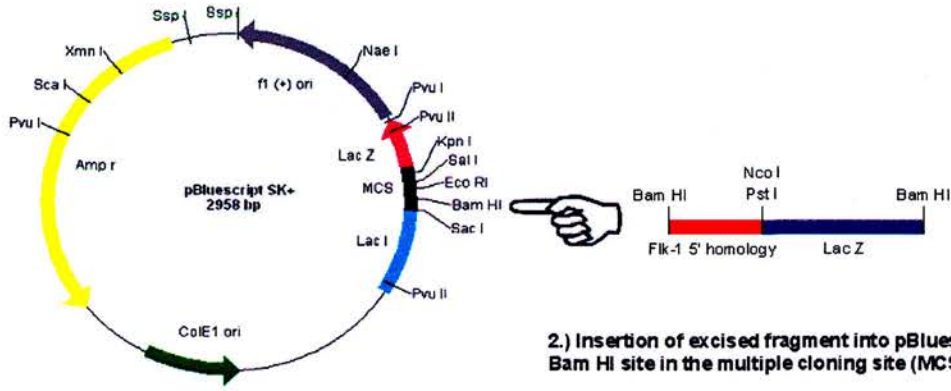


6.) The *flk-1*/HPRT plasmid was linearised for electroporation with the single cutting Not I enzyme.

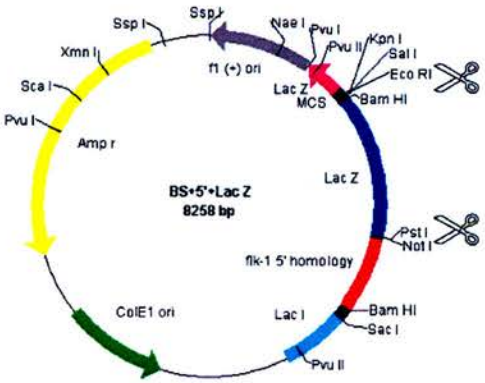
Appendix Aii:
Cloning Strategy: flk-1/GFP Vector Construction



1.) Bam HI digest to excise 5' homology and Lac Z reporter

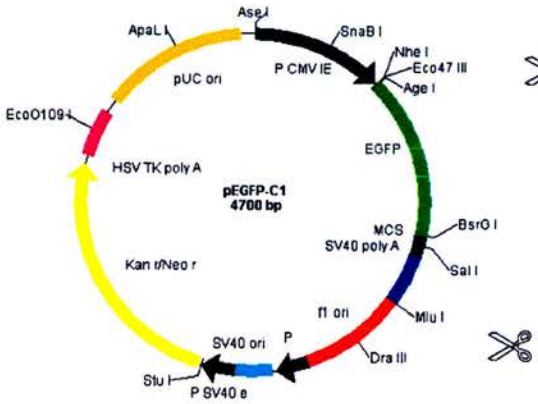


2.) Insertion of excised fragment into pBluescript SK+ at Bam HI site in the multiple cloning site (MCS).



3.) Excision of Lac Z reporter using Eco RI and Pst I enzymes.

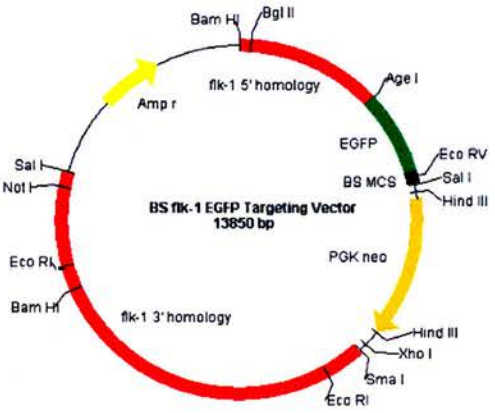
4.) The Eco RI and Pst I overhangs were blunted, in preparation for a blunt-end insertion of the EGFP cDNA.

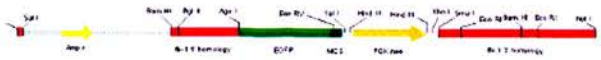


5.) Concurrently, the EGFP cDNA was excised from the pEGFP-C1 plasmid with a Eco 47III and Mlu I digest.

6.) The ends of the isolated plasmid were blunted.

7.) The isolated EGFP cDNA was used to replace the excised Lac Z reporter to complete the *flk-1*/GFP plasmid.





8.) The *flk-1*/GFP plasmid was linearised for electroporation with the single cutting Not I enzyme.

Appendix B:

Statistical Analysis of the significance of EB disaggregation techniques and EBs age on cell survival and differentiation in methylcellulose culture.

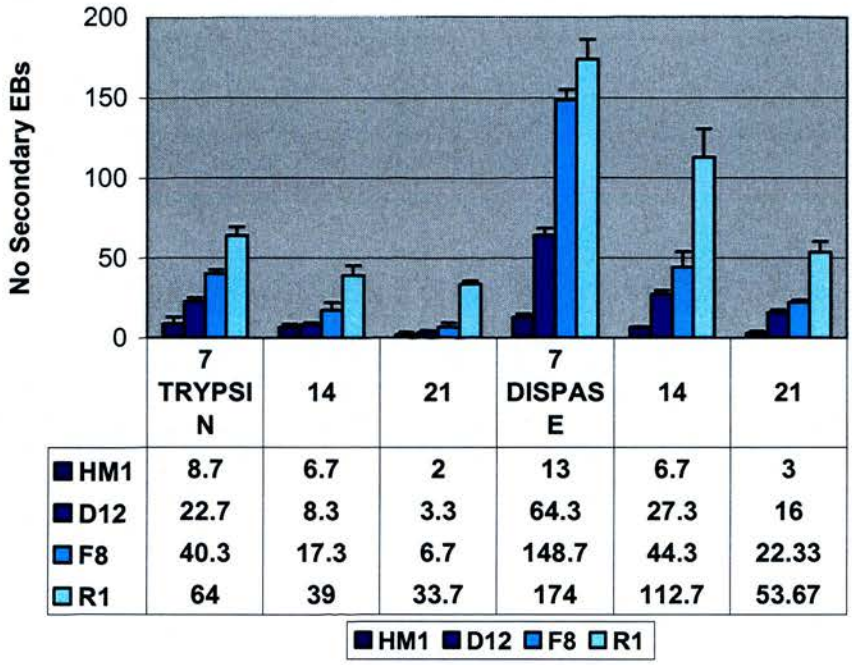
1) 3 day and 4 day Embryoid Bodies

The first data set consists of counts of 3 day and 4 day Embryoid Bodies (EBs) that have been cultured for different lengths of time (7, 14, 21 days) under two treatment regimes (trypsin, dispase) and are derived from 4 different cell lines (HM1, R1, D12, F8). All observations are in triplicate.

1.1) 3 day Embryoid Bodies

The raw mean counts and standard error bars for day 3 EBs are shown in Fig 1 below.

Figure 1: Numbers of 3 day Embryoid Bodies



Statistical Analysis

On the original scale there is a dependency between the variance and the mean, ie. the variation between triplicate counts increases as the mean value increases. Before analysis the counts were transformed onto the log scale to remove this dependency. Analysis of variance was used to look at the effects of treatment (trypsin, dispase), cell line (HM1, R1, D12, F8) and day (7, 14, 21) on the numbers of EBs observed. The final ANOVA table is shown below (data on log scale). No interactions were significant. All main effects were significant.

Analysis of variance

ANOVA: leb3

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
treat	1	20.7907	20.7907	36.55	<.001
cell_line	3	77.7428	25.9143	45.56	<.001
day	2	26.7002	13.3501	23.47	<.001
Residual	65	36.9706	0.5688		
Total	71	162.2043			

Tables of means

Variate: leb3

Grand mean 2.90

treat	disp	tryp
	3.44	2.37

cell_line	d12	f8	hm1	r1
	2.76	3.34	1.34	4.19

day	7.	14.	21.
	3.60	3.00	2.12

Standard errors of means

Table	treat	cell_line	day
rep.	36	18	24
d.f.	65	65	65
e.s.e.	0.126	0.178	0.154

Conclusions

The number of day 3 EBs is affected by all factors in the experiment. The F statistics in the ANOVA table are highly significant ($p<0.001$) in all cases. When individual levels of a factor are compared with each other (eg. one cell line compared to another) the significance is generally lower.

1. Cell line: The differences between cell lines are consistent across the other factors. R1 has the highest counts followed by F8, D12 and HM1. Overall the difference between cell lines is significant at the 0.1% level. Individually all cell lines differ from each other ($p<0.05$).

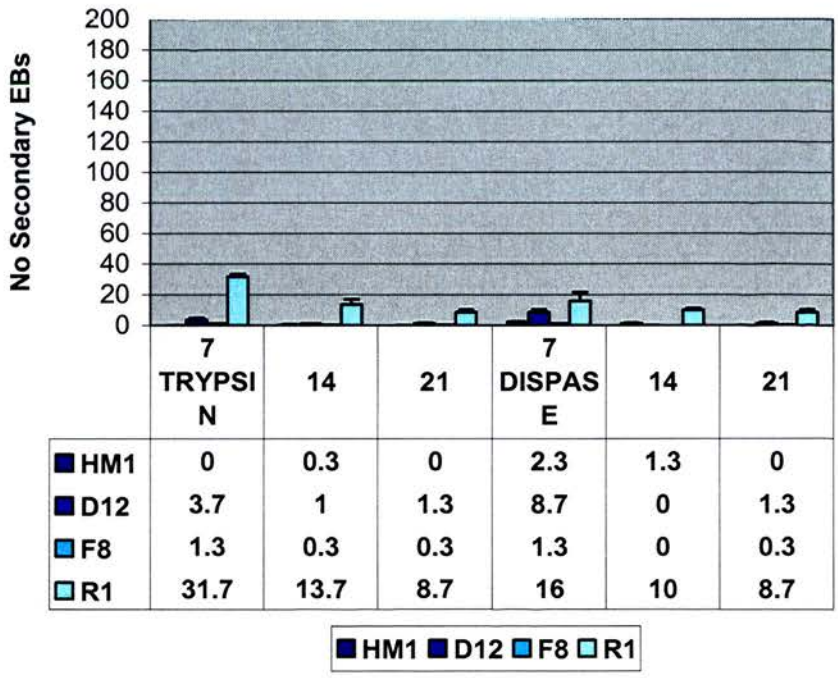
2. Day: The numbers of EBs decrease as day increases from 7 to 14 to 21. Overall the difference is significant at 0.1%. Individually all days differ from each other ($p<0.05$).

3. Treatment: Trypsin produces consistently higher counts than dispase. The difference is significant at the 0.1% level. In this case the F statistic is used because the factor has only two levels.

1.2) 4 day Embryoid Bodies

The counts for Day 4 EBs are uniformly lower than those for Day 3 EBs across all treatment/cell line/day combinations (see Fig 2 below). There are 26 zero counts out of 72 counts for individual replicates. Only R1 consistently produced reasonably high mean numbers of EBs across all treatment combinations (8.7-31.7). F8 and HM1 produced means in the range 0.0-2.3 and D12 in the range 0.0-8.7. Only cell line R1 has an average count greater than zero for all days and both treatments. The very low counts make analysis more difficult.

Figure 2: Numbers of 4 day embryoid bodies



	7 TRYPSIN	14	21	7 DISPASE	14	21
HM1	0	0.3	0	2.3	1.3	0
D12	3.7	1	1.3	8.7	0	1.3
F8	1.3	0.3	0.3	1.3	0	0.3
R1	31.7	13.7	8.7	16	10	8.7

Statistical Analysis

The data were transformed onto the log scale to stabilise the variance. Some treatment groups heave zero variance because three all replicates produced no EBs. The treatment combinations that were more successful, eg. R1 x trypsin, tend to have high variances. Analysis of Variance was performed on the transformed data, as for Day 3 EBs. In this case all the interactions were significant and no general conclusions could be drawn. The effect of treatment (trypsin or dispase) varies significantly across days (7/14/21) and between cell lines (R1, D12, HM1, F8).

Analysis of variance

Variate: leb4

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
cell_line	3	171.2762	57.0921	75.54	<.001
treat	1	0.1616	0.1616	0.21	0.646
day	2	27.4326	13.7163	18.15	<.001
cell_line.treat	3	15.2261	5.0754	6.72	<.001
cell_line.day	6	18.7489	3.1248	4.13	0.002
treat.day	2	5.3400	2.6700	3.53	0.037
cell_line.treat.day	6	11.4794	1.9132	2.53	0.033
Residual	48	36.2793	0.7558		
Total	71	285.9442			

Tables of means

ANOVA: leb4

Grand mean 0.08

cell_line	d12	f8	hm1	r1
	0.20	-1.23	-1.20	2.56

treat	disp	tryp
	0.13	0.03

day	7.	14.	21.
	0.95	-0.29	-0.41

cell_line	treat	disp	tryp
d12		-0.15	0.54
f8		-1.36	-1.09
hm1		-0.37	-2.04
r1		2.40	2.72

cell_line	day	7.	14.	21.
d12		1.68	-1.10	0.02
f8		-0.27	-1.90	-1.50
hm1		-0.72	-0.60	-2.30
r1		3.11	2.44	2.13

treat	day	7.	14.	21.
disp		1.38	-0.50	-0.49
tryp		0.53	-0.08	-0.34

	treat	disp			tryp		
cell_line	day	7.	14.	21.	7.	14.	21.
d12		2.14	-2.30	-0.27	1.23	0.10	0.31
f8		-0.27	-2.30	-1.50	-0.27	-1.50	-1.50
hm1		0.87	0.31	-2.30	-2.30	-1.50	-2.30
r1		2.77	2.30	2.12	3.46	2.57	2.14

Standard errors of differences of means

Table	cell_line	treat	day	cell_line treat
rep.	18	36	24	9
d.f.	48	48	48	48
s.e.d.	0.290	0.205	0.251	0.410

Table	cell_line day	treat day	cell_line treat day
rep.	6	12	3
d.f.	48	48	48
s.e.d.	0.502	0.355	0.710

Conclusions

For day 3 EBs dispase produces significantly more EBs than trypsin ($p<0.001$) and the number falls significantly from 7 to 14 and then to 21 days ($p<0.05$). The R1 cell line produces the most EBs followed by F8, D12 and HM1. The differences between individual lines are significant ($P<0.05$).

For day 4 EBs the interactions between treatment, day and cell line are all significant and no general statements can be made about the significance of these effects. The trend is for numbers of EBs to decrease as day increases from 7 to 21 and for R1 to produce the highest numbers of EBs, as was the case for day 3 EBs. There is no clear difference between trypsin and dispase, unlike day 3 EBs.

Comments

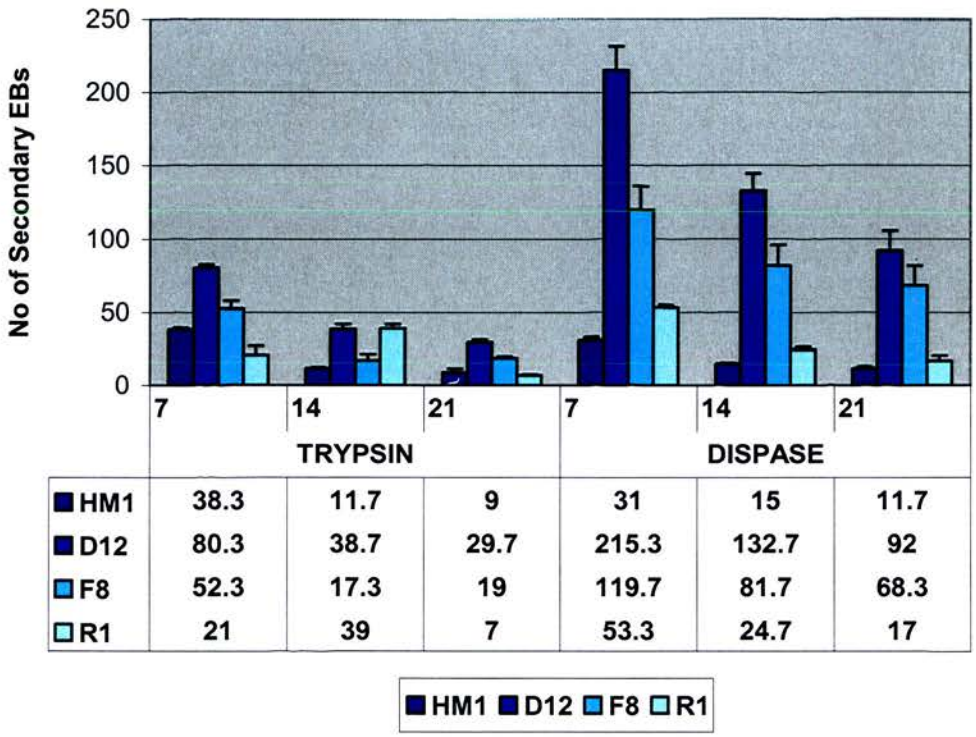
These data can also be analysed assuming that the cell lines are "random" – i.e. that the individual cell lines are not of interest but merely represent a sample from a range of cell lines. If an analysis on this basis is performed the results are similar. For day 3 EBs the effects of treatment and day are significant as they were for the ANOVA. For day 4 EBs only the effect of day is significant. The overall conclusions remain the same.

2) 2.75 and 3.5 day EBs

The data set is of the same form as in (**1**) except for the age of the EBs (see Figures 3 & 4 and ANOVA tables below). The pattern of the responses of the cell lines to the treatments over time is similar and the same type of statistical analysis was performed.

2.1) 2.75 day Embryo Bodies

Figure 3 Day 2.75 Embryo Bodies



	TRYPSIN			DISPASE		
	7	14	21	7	14	21
HM1	38.3	11.7	9	31	15	11.7
D12	80.3	38.7	29.7	215.3	132.7	92
F8	52.3	17.3	19	119.7	81.7	68.3
R1	21	39	7	53.3	24.7	17

Results

For 2.75 day EBs there is a significant interaction between treat and cell line. This is due to the fact that HM1 has almost the same number of EBs under Trypsin and

Dispase compared with R1, D12 and F8, which have more EBs on the dispase treatment than on the trypsin treatment. Figure 3 shows this effect clearly. The differences between numbers of EBS for R1, D12 and F8 are significant at the 5% level when tested individually. The effect of day is also significant ($p<0.001$). The numbers of EBs decreases approximately linearly with time.

ANOVA: leb2.75

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
treat	1	10.86905	10.86905	152.36	<.001
day	2	13.00004	6.50002	91.11	<.001
cell_line	3	31.04519	10.34840	145.06	<.001
treat.cell_line	3	2.79274	0.93091	13.05	<.001
Residual	62	4.42303	0.07134		
Total	71	62.13005			

Tables of means

Variate: leb27_5

Grand mean 3.497

treat	disp	tryp
	3.886	3.108

day	7.	14.	21.
	4.063	3.390	3.039

cell_line	d12	f8	hm1	r1
	3.901	2.923	2.802	4.362

treat	cell_line	d12	f8	hm1	r1
disp		4.440	3.321	2.866	4.915
tryp		3.362	2.525	2.738	3.809

Standard errors of differences of means

Table	treat	day	cell_line	treat cell_line
rep.	36	24	18	9
d.f.	62	62	62	62
s.e.d.	0.0630	0.0771	0.0890	0.1259

If an analysis assuming random cell lines is performed then the effects of treatment and day are both significant ($p<0.01$). Dispase is superior to trypsin and the numbers of EBs decreases approximately linear as the number of days increases from 7 to 21.

Conclusions

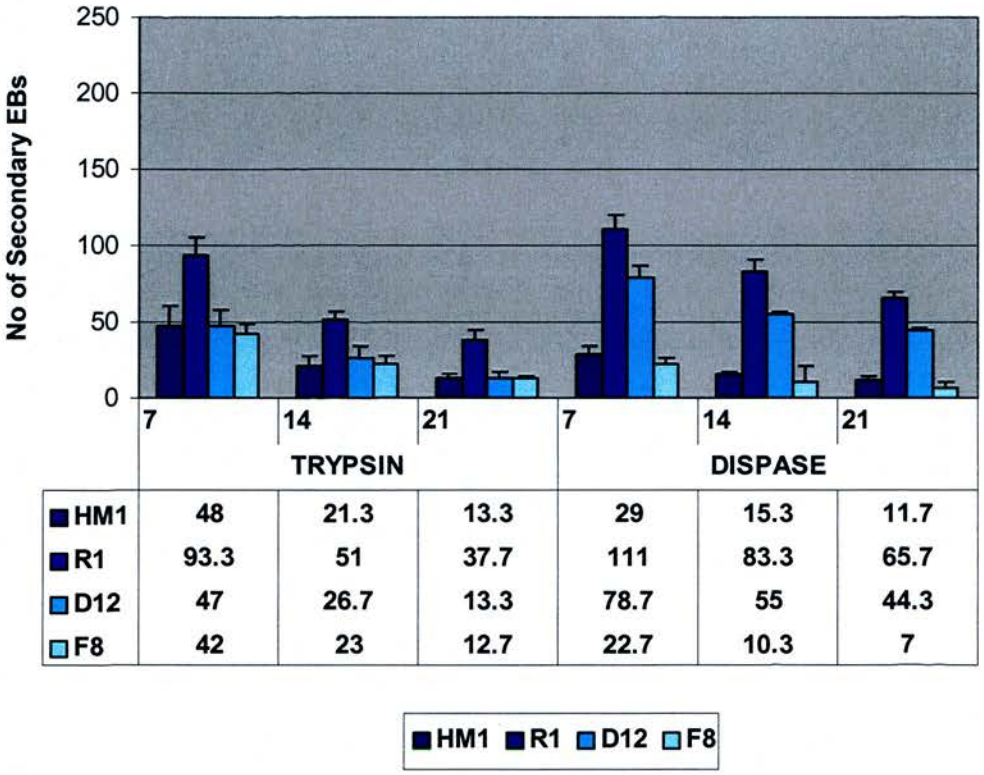
As for day 3 and day 4 EBS there is a clear effect of time. Numbers of EBS decrease as days increase from 7 to 21. The effect of treatment is not so simple. The treatment effect (trypsin versus dispase) is very small for one cell line (HM1) but dispase is consistently (and statistically significantly) superior for the other 3 (R1, D12, F8). If the analysis with random cell lines is used then this is not an issue (it is taken into account as random variation) and the effect of treatment is significant overall.

2.2) 3.5 day Embryo Bodies

Results

For 3.5 day EBs there is a significant interaction between treatment and cell line. There is no consistency between the treatments. Trypsin is superior for F8 and HM1. Dispase is superior for R1 and D12, which have higher numbers of EBs on both treatments than the other 2 cell lines. Again the effect of day is significant and consistent – an approximately linear decrease over time.

Figure 4: Day 3.5 embryo bodies



An analysis assuming random cell lines produces a significant difference only between days. There is no significant treatment effect.

Conclusion

The only consistently significant effect is that of day. Dispase is still superior to Trypsin for some cell lines but the effect is less consistent than it was for 3 and 4 day EBs.

3) 2.75 and 3.5 day haematopoietic colonies

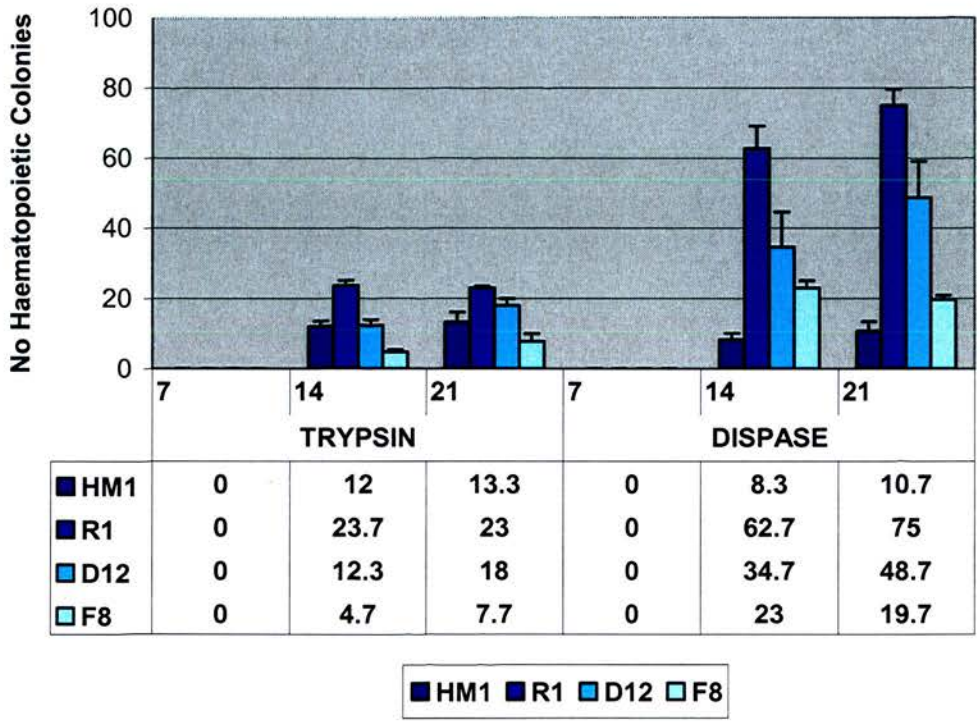
Some EBs turn into haematopoietic colonies. Figures 5 and 6 below show the numbers of haematopoietic colonies at 2.75 and 3.5 days for the same set of

treatments and cell lines described above. The same analysis was used as for EBs and, as before, the data were transformed onto the log scale to stabilise the variance.

3.1) 2.75-day haematopoetic colonies

There is a significant interaction between treatment and cell line. Three cell lines produce more haematopoietic colonies (HCs) on dispase, namely R1, D12 and F8. One cell line, HM1, produces more HCs on trypsin. The effect of day is also significant ($p<0.05$). There are more HCs on day 21 than on day 14.

Figure 5: 2.75 day haematopoeitic colonies



	TRYPSIN			DISPASE		
	7	14	21	7	14	21
HM1	0	12	13.3	0	8.3	10.7
R1	0	23.7	23	0	62.7	75
D12	0	12.3	18	0	34.7	48.7
F8	0	4.7	7.7	0	23	19.7

ANOVA: lh2.75

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
treatment	1	6.83949	6.83949	70.57	<.001
day	1	0.43667	0.43667	4.51	0.040
cell_line	3	14.95083	4.98361	51.42	<.001
treatment.cell_line	3	4.84846	1.61615	16.67	<.001
Residual	39	3.77992	0.09692		
Total	47	30.85537			

Tables of means

Variate: lh2.75

Grand mean 2.895

treatment	disp	tryp
	3.272	2.517

day	14.	21.
	2.800	2.990

cell_line	d12	f8	hm1	r1
	3.158	2.391	2.347	3.684

treatment	cell_line	d12	f8	hm1	r1
disp		3.631	3.051	2.187	4.221
tryp		2.684	1.730	2.508	3.148

Standard errors of differences of means

Table	treatment	day	cell_line	treatment cell_line
rep.	24	24	12	6
d.f.	39	39	39	39
s.e.d.	0.0899	0.0899	0.1271	0.1797

Least significant differences of means (5% level)

Table	treatment	day	cell_line	treatment cell_line
rep.	24	24	12	6
d.f.	39	39	39	39
l.s.d.	0.1818	0.1818	0.2571	0.3636

If cell line is treated as random then the effect of day is no longer significant but the effect of treatment is significant ($p<0.01$). Dispase produces significantly more HCs than Trypsin when the variation between cell lines is seen as another source of random experimental error rather than a systematic effect.

3.2) 3.5 day haematopoetic colonies

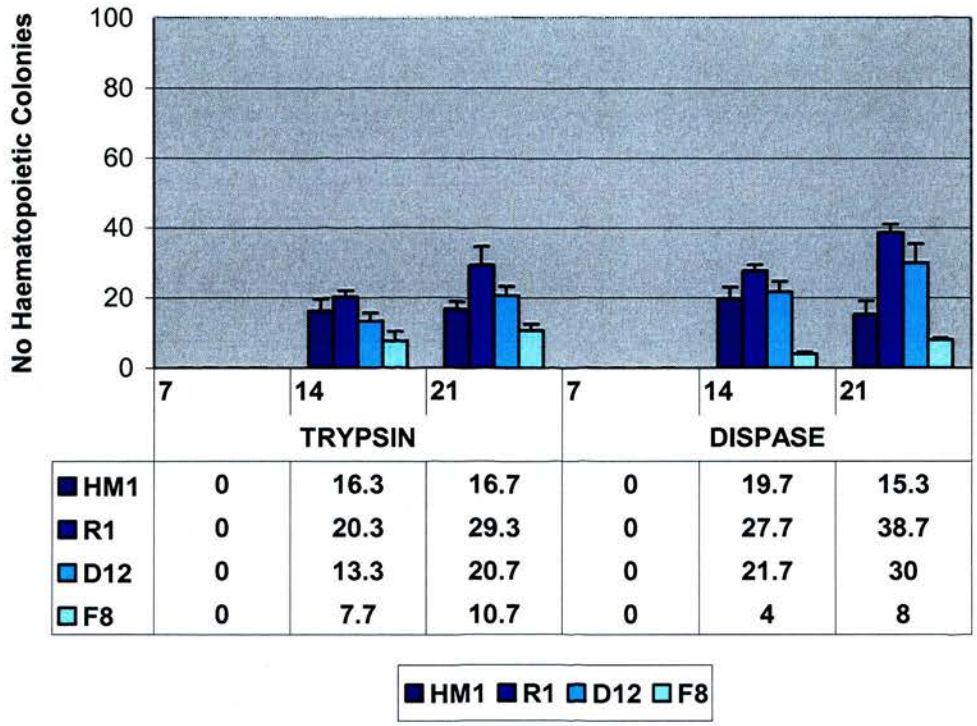
The results of the ANOVA are very similar to those for 2.75 day HCs (see table below). There is a significant interaction between treatment and cell line. Dispase is superior only for D12, it is marginally better for R!, F8 produces more HCs on trypsin and HM1 produces almost identical results on each treatment (see Figure 6 below). Again there is a significant effect of day ($p<0.01$). There are more HCs on day 21 than on day 14.

If cell line is treated as a random effect then there is a significant effect of day ($p<0.01$) but no other significant effects. This is in contrast to the results for 2.75 day HCs.

Conclusion

In this case the conclusions are dependent upon the assumptions about the cell line effect. If cell lines are assumed fixed then for both 2.75 and 3.5 day HCs there is a significant effect of day (more HCs on day 21 than on day 14) but no consistent treatment effect because this varies across cell lines. If we assume cell lines to be a random effect then the results for 2.75 and 3.5 day HCs are not consistent. There is a significant effect of treatment on day 2.75 (dispase is superior to trypsin) but no treatment effect on day 3.5. Conversely there is no effect of day on 2.75 day HCs but there is a significant day effect (day 21 has more HCs than day 14) on 3.5 day HCs.

Figure 6: 3.5 day haematopoeitic colonies



HM1	0	16.3	16.7	0	19.7	15.3
R1	0	20.3	29.3	0	27.7	38.7
D12	0	13.3	20.7	0	21.7	30
F8	0	7.7	10.7	0	4	8

ANOVA: lh3.5

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
treatment	1	0.0919	0.0919	0.89	0.352
day	1	1.0052	1.0052	9.70	0.003
cell_line	3	13.0225	4.3408	41.91	<.001
treatment.cell_line	3	1.2177	0.4059	3.92	0.015
Residual	39	4.0395	0.1036		
Total	47	19.3769			

Tables of means

Variate: lh3.5

Grand mean 2.759

treatment	disp	tryp
	2.803	2.715

day	14.	21.
	2.614	2.904

cell_line	d12	f8	hm1	r1
	2.997	1.921	2.787	3.330

treatment	cell_line	d12	f8	hm1	r1
disp		3.208	1.719	2.799	3.484
b		2.786	2.123	2.775	3.176

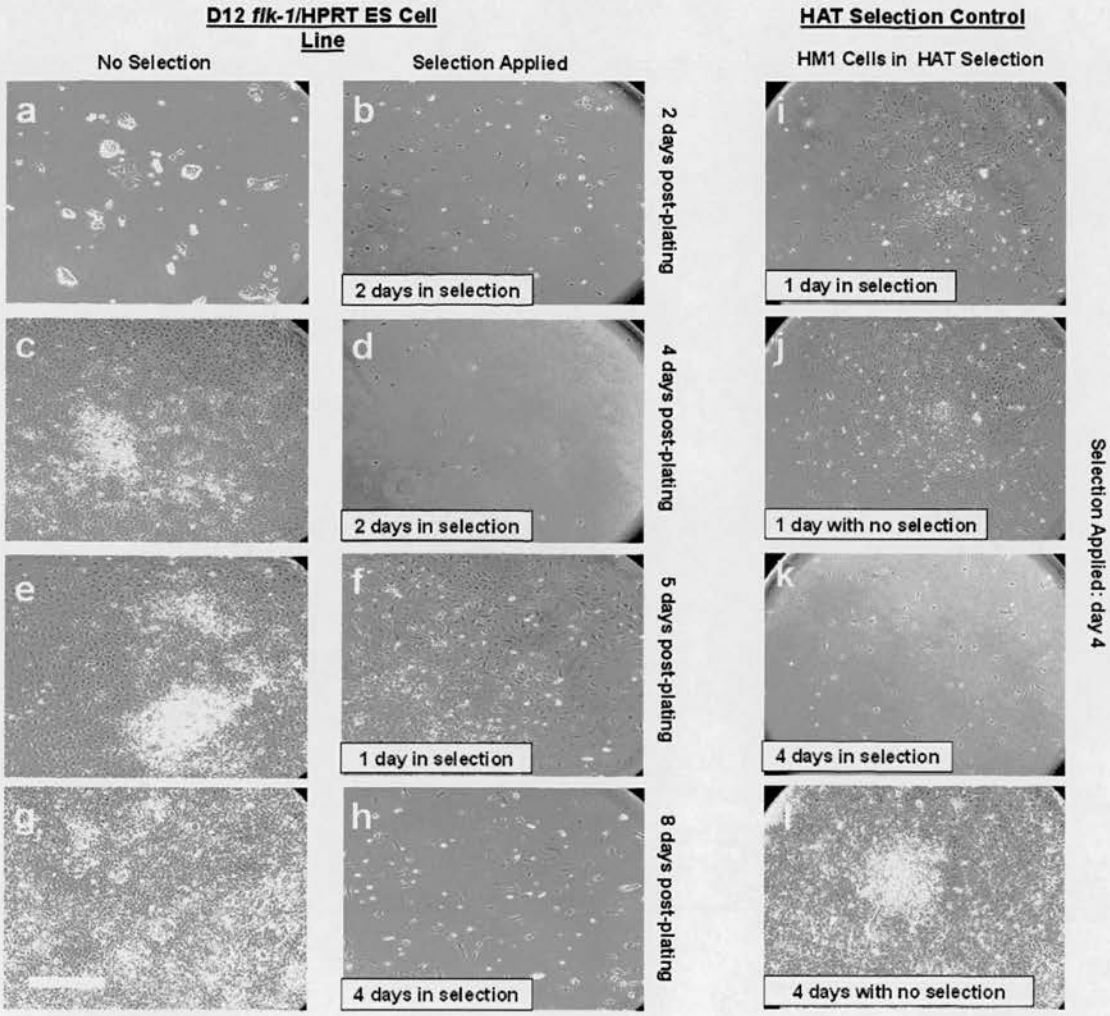
Standard errors of differences of means

Table	treatment	day	cell_line	treatment cell_line
rep.	24	24	12	6
d.f.	39	39	39	39
s.e.d.	0.0929	0.0929	0.1314	0.1858

Least significant differences of means (5% level)

Table	treatment	day	cell_line	treatment cell_line
rep.	24	24	12	6
d.f.	39	39	39	39
l.s.d.	0.1879	0.1879	0.2658	0.3758

Appendix C:
Supplementary photographs of repeats of the differentiation trial shown in Figure 6.2



*Cell number and morphology of flk-1/hprt ES cells growing with (**b, d, f, h**) and without (**a, c, e, g**) HAT selection. HAT selection was applied at various timepoints: at the time of plating on collagen IV: day 0 (**b**), and at 2 (**d**) and 4 days (**f, h**) after plating. Cultures were maintained until 8 days after plating when the control plates were fully confluent (**g**). HM1 ES cells were used as a negative control to assess the level of cell death expected from a completely HPRT deficient ES cell line (**i, j**). Cells maintained under identical growth conditions in the absence of any selection are shown in **j** and **l**. Scale bar represents 200μm.*

Appendix D:
Supplementary photographs of repeats of the differentiation trial shown in Figure 6.3

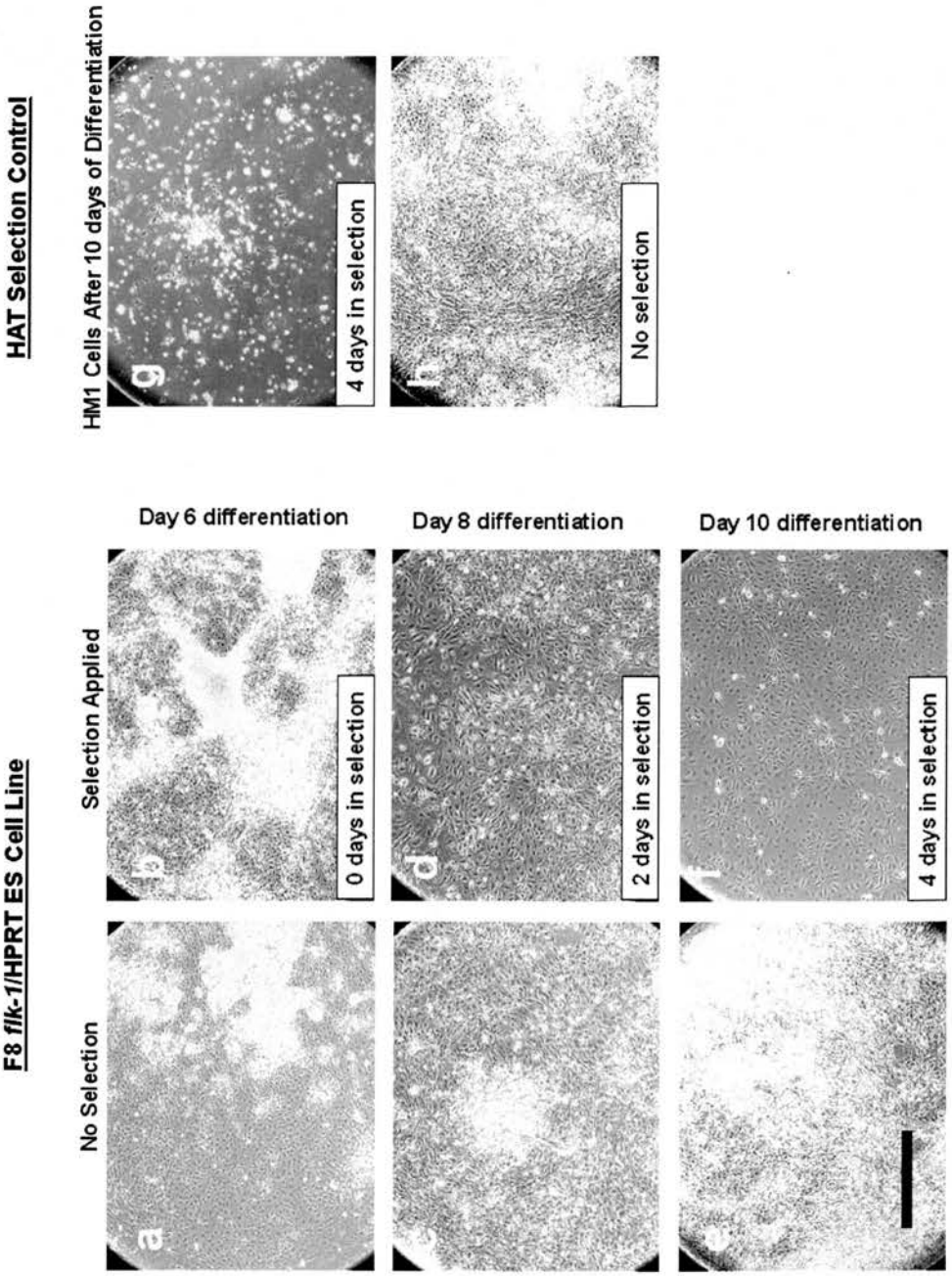


Figure 6.3 *Course of selection and cell death after HAT selection is applied at 6 days of differentiation of flk-1/HPRT ES cells on collagen IV. Cells in and out of selection are shown at 6 (**a, b**), 8 (**c, d**) and 10 (**e, f**) days of differentiation. HAT sensitive HM1 ES cells that have been placed in selection for 4 days are shown (**g**) alongside those that have not been placed under HAT selection (**h**). Scale bar represents 200μm.*

Appendix E:
Immunohistochemistry secondary antibody only controls

All immunohistochemical analyses were accompanied with secondary antibody controls to ensure that non-specific binding of the secondary antibody was not responsible for the detectable staining observed in test samples.


The End